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Immunocytochemical light microscopy and transmission electron microscopy study of growth hormone secreting cells in the porcine anterior pituitary

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Neuroscience

Program of Study Committee:
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Ames, Iowa

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Signatures have been redacted for privacy

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GENERAL INTRODUCTION

Literature Review

Growth hormone action in the central nervous system

1. Growth hormone (GH)

Endocrine regulation in the brain is important for body growth and metabolism both in human and domestic animals. Growth hormone has growth-promoting effects and metabolic actions that are important in many species and acts directly or indirectly on virtually every tissue in the body. The main targets of GH actions are liver, muscle, and bone where this action is partially mediated through an increase in insulin-like growth factor-I (IGF-I), a highly conserved to protein. GH also has direct effects in many tissues coordinating with locally generated/circulating IGF-I to enhance growth in animals. It is well known that GH treatment induces IGF-I and stimulates IGF-I expression in the liver and other tissues (Sato et al., 1993; Harel et al., 1992).

The biological events of GH are initiated by hormone binding to its cell surface receptor and trigger gene expression for long-term changes that are responsible for the stimulation of growth. The complex between hGH and the hGHR is one of the ligand-receptor pairs of the hematopoietic cytokine family that transducer signals through their receptors via sequential dimerization mechanism (Cunningham et al., 1991). GH receptor (GHR) is expressed in many tissues, including liver, heart, fat, testis, skeletal muscle, intestine, kidney, pancreas, and brain (Noblet et al., 1992; Frick et al., 1990; Harvey et al., 1993). Especially brain is considered as an important target tissue for GH supported by increasing direct evidence. High-resolution autoradiography (Zhai et al., 1994; Pellegrini et al., 1996) and immunocytochemistry (Lobie et al., 1993) and in situ hybridization (Smith et

al., 1989; Baumbach et al., 1989) have been used to confirm GHR expression in the central nervous system (CNS)-hypothalamus and hippocampus in rodents and human. There is increasing evidence that GHRs are involved in a short loop feedback regulating GH secretion within the hypothalamus. GHR expression localized in perivetricular nucleus (PVN) somatostatin (SS) neurons is consistent with the GH feedback inhibition to decrease GHRH expression (Burton et al., 1992). Decreasing hypothalamic GHR expression in hypophysectomy or in dwarf rat with specific GH deficiency and restored their expression by treating them with exogenous GH suggested that central GHR expression is also sensitive to GH regulation (Minami et al., 1993; Bennett et al., 1995).

A notable physiological effect of GH in the CNS is inhibition of its own release, as part of an autofeedback circuit (Tannenbaum et al., 1980; Robinson et al., 1993; Bennett et al., 1995). Studies in chronically cannulated rats demonstrated that GH inhibits its own secretion rapidly blocking spontaneous pulsatility (Willoughby et al., 1980; Clark et al., 1988).

2. Feedback mechanism of Growth hormone releasing hormone and Somatostatin

There is little evidence of a direct effect of GH to inhibit its own release at the pituitary (Richman et al., 1981), rather that it acts in the hypothalamus to target the peptidergic systems regulating GH release although expression of GHRs is detected in the pituitary gland. This may implicate that time scale of GH feedback. In most species, a pulsatility pattern of GH secretion is regulated by tightly controlled feedback pathways (Chihara et al., 1981; Conway et al., 1985; Lanzi et al., 1992). Two hypothalamic peptide hormones highly involved in the feedback loop are growth hormone releasing hormone (GHRH) and somatostatin (SS). This episodic pulsatility reflects a balanced alteration in two

neuroendocrine systems regulating GH release by a positive/negative feedback loop. GHRH is released from arcuate neurons in the hypothalamus and transported through the portal blood vessels into the pituitary gland, where it stimulates GH release from somatotrophs (GH-secreting cells in the pituitary gland). Negative feedback is mediated by the release of somatostatin (SS) from hypothalamic neurons that act to inhibit GH release (Chihara et al., 1981; Chomczynski et al., 1988).

GHRH is a peptide hormone synthesized and released from the hypothalamic arcuate nucleus (ARC) that stimulates GH secretion from pituitary somatotroph cells. The pituitary cell membrane has specific, high-affinity binding sites for GHRH. Binding GHRH to its receptor stimulates adenylate cyclase, resulting increased adenosine 3'5'-cyclic monophosphate (cAMP) production, and indicating that the G_s protein is an intermediate in GHRH action. Because cAMP is an important second messenger for GHRH signaling, GHRH and cAMP stimulate pituitary GH secretion, facilitate GH gene expression, and increase the proliferation of cultured pituitary somatotroph cells (Chen et al., 1995; Mayo et al., 1995).

SS or somatotrophin-releasing inhibiting factor (SRIF) is a 14 amino acid-containing peptide hormone primarily expressed in the hypothalamus. A major physiological function of SS is to inhibit GH release and maintain the pulsatile secretion of GH. SS inhibits GH release by activating the receptor subtype $sstr_2$ to inhibit Ca^{2+} conductance and Ca^{2+} influx in somatotrophs (Reisine et al., 1995; Fuji et al., 1994).

The GH feedback loop by GHRH and SS on pulsatile GH release is maintained with time. GHRH expression is increased in the case of GH deficiency, whereas GH treatment reverses changes (Chomczynski et al., 1988). Conversely, SS expression in the

hypothalamus is decreased after hypophysectomy, whereas excess GH stimulates hypothalamic SS synthesis and release (Chihara et al., 1981; Rogers et al., 1988). The main physiological role of GH feedback on GHRH is to regulate the GH reserve for a much longer time by maintaining somatotroph proliferation. Although local generation of IGF-I in response to GH secretion in the CNS is considered to be important, changes in GHRH and SRIF are readily observed with GH, but not with IGF-I alone, which implicates sites of direct feedback for GH and not secondary to peripheral IGF-I generation (Lanzi et al., 1992; Sato et al., 1993).

3. *Growth hormone secretagogues (GHSs)*

Pulsatile GH secretion from the pituitary somatotrophs was thought to be regulated by episodic changes only in two hypothalamic hormones, GHRH and SRIF. However, the discovery of synthetic GHRP by Bowers et al. (1984) and several nonpeptidyl GHSs including L-692,429, L-692,585, and MK-0677 that also act to enhance GH release in several animal species has brought an emerging perspective in the endogenous regulation of GH secretion (Clark et al., 1989; Bowers et al., 1990; Malozowski et al., 1991). GHRP-6 stimulates pulsatile GH release through the activation of a receptor distinct from the SRIF and GHRH receptor, which belongs of the family of seven-transmembrane receptor coupled to GTP binding protein to up-down-regulate cAMP level controlling GH secretion. Peptidyl and nonpeptidyl GHS receptors cloned in the anterior pituitary and hypothalamus are activated by a mediator, a phosphoinositol-protein kinase C intracellular pathway that induces intracellular Ca^{2+} release and depolarization (Cheng et al., 1991), leading to exocytosis of GH-containing secretory vesicles in the pituitary gland.

GHSs may have dual action to stimulate endogenous GHRH release and suppress endogenous somatostatin release, which implies GHSs require the presence of a functional hypothalamus (Bowers et al., 1991). In situ hybridization studies in monkey and rat brains show that GHS-R is expressed in arcuate neurons, suggesting that GHS-R ligand stimulates these neurons directly as a GHRH releaser that induces GH secretion from the somatotrophs (Smith et al., 1997). GHS and GHRH have a synergistic effect and even very low GHS doses potentiate the GHRH-induced GH rise in human and animals (Bowers et al., 1990). Hypothalamopituitary disconnection (functional stalk section) fails to respond to GHSs for optimal GH release. Experiment in hypothalamic stalk-sectioned pigs showed that GHS-induced GH release is blocked and synergistic action of GHRH and GHS is absent. In swine, hypothalamic-pituitary stalk transaction significantly decreased the GH response to the GHS L-692,585, but the GH response to combined GHRH/GHS bolus was similar to the GH rise in the intact animals (Hickey et al., 1996). These results proposed that mediation of GHS action through hypothalamic release of GHRH. Electophysiological studies *in vivo* show that neurosecretory neurons in the hypothalamic arcuate nucleus are excited by GHSs and are inhibited during electrical stimulation of periventricular nucleus (Dickson et al., 1993a, 1995b). Also, after intravenous injection of somatostatin, secretagogue-responsive cells are inhibited. Thus, it would appear that a subpopulation of the arcuate cells activated by GHSs is inhibited by central somatostatin action (Zheng et al., 1997). These important studies link the action of GHS-R ligands with two endogenous regulators of GH release, GHRH and somatostatin, which are important to generate the rhythm of GH pulsatility.

Interestingly, studies in animals have suggested that GHSs may have widespread effect in the brain and interfere with secretion of neuropeptide Y and dopamine, which leads to up-

regulation of adrenocorticotropin (ACTH), cortisol, and prolactin secretion. For example, the nonpeptide GHRP analog L-692,429 powerfully stimulated prolactin secretion from pituitary somatomammotrope cells (Adams et al., 1998). This study implies that GHS might include mastopathy, galactorrheas, and/or a loss of libido. A most important reason prompting GHS research is its benefit of oral administration to release GH (Bowers et al., 1997). Therefore, it would be useful in clinical practice for diagnostic and therapeutic purpose as well as potential in regulating GH secretion in farm animals.

4. *Neuropeptide Y (NPY) & Leptin*

NPY has been implicated as a regulator in the control of appetite, body weight gain, and obesity, and is also involved in central mechanism of GH release. NPY expression is GH sensitive and may be the primary target for GH feedback in the ARC (Chan et al., 1994). Previous studies showed that ARC NPY mRNA is reduced in GH-deficient dwarf rats and this deficiency is corrected by GH administration (Bennett et al., 1995). So, ARC NPY is stimulatory effecter on somatostatin and inhibitory effecter on GHRH. Therefore, GH action of these cells might be involved in food intake activity in the GH/IGF-I axis affecting food utilization. Recently, NPY has been considered as a mediator of the effects of GHSs, and leptin action.

Leptin is newly discovered a 143-amino-acid peptide hormone product of obese gene (*ob*), secreted by adipocytes that regulate food intake and energy expenditure (Zhang et al., 1994; Kalra et al., 1996). Leptin has recently been shown to play a stimulatory role on GH secretion by mediating NPY in the hypothalamic area (Eva et al., 1998). Alternatively, leptin and NPY could act through parallel pathway to change GH release with NPY, overcoming

the stimulatory effect exerted by leptin on plasma GH level. One of the leptin receptors, *ob*-Rb is responsible for the leptin signaling in the brain and expressed in the hypothalamic area: median eminence (ME), arcuate nucleus (ARC), ventromedial nucleus (VMN), and dorsomedial nucleus (DMN). The main physiological role of leptin is to increase energy expenditure and decrease in food intake after signal increasing adiposity to the brain. Also, a study of leptin action on the reproduction demonstrates that leptin stimulates the reproductive endocrine system and suggests that leptin may serve as a permissive signal to the reproductive system of normal animals (Ilona et al., 1996).

5. *Ghrelin*

Ghrelin is the newest player in the endogenous regulation of GH secretion. Ghrelin, a novel 28 amino acid peptide has recently been purified from rat stomach and subsequently cloned in pancreas, kidney, placenta, pituitary, and hypothalamus of rat and human (Kojima et al, 1999; Date et al., 2000; Mori et al., 2000; Gualillo et al., 2001;Korbonits et al., 2001, Volante et al., 2002). An important physiological role of ghrelin is the endogenous ligand for the GHS-R to induce directly GH release from pituitary somatotrophs (Kojima et al., 1999). The acylation of the peptide had been supposed critical to cross the blood-brain barrier but is also essential for binding the GHS1a receptor and for its GH-releasing and other endocrine actions (Kojima et al., 1999; Bednarek et al., 2001; Muccioli et al., 2001). It is still thought that GHS, ghrelin –induced GH secretion is antagonized by a hypothalamic-somatostatin action and involves a GHRH-mediated pathway (Arvat et al., 2001; Gurd et al., 2001; Tannenbaum et al., 2001). By using immunocytochemical detection method, ghrelin has been detected in the hypothalamic arcuate nucleus (ARC) and in the stomach (Kojima et al.,

2001). The hypothalamic ARC is a major hypothalamic site for regulation of eating behavior and body weight since it contains neuropeptide Y (NPY), agouti-related protein (AGRP), cocaine, amphetamine-regulated transcript (CART), and pro-opiomelanocortin (POMC) (Wood et al., 1998; Abbott et al., 2001). Therefore, this hypothalamic ghrelin has important physiological roles in GH secretion and energy balance. The data showed that exogenous ghrelin induces adiposity in rodents by stimulating increase in food intake and a reduction in fat utilization based on the hypothalamic signaling (Shintani et al., 2001). Adipogenic and orexigenic effects of ghrelin are dissociated from its GH secretion effects and rather are associated with a specific central mechanism of neurons mediated by leptin whose regulation and biological effect are opposed to those of ghrelin. The further understanding of central network and function of ghrelin will enlighten the general frame for hypothalamic machinery in metabolism regulation.

Pituitary gland

1. Anatomy and development

The pituitary gland is a critical component of the neuroendocrine system that is essential for the maintenance of homeostasis, metabolism, reproduction, lactation, and growth. Maintenance of normal body function requires integration of the endocrine system with the nervous and immune systems (Ojeda and Griffin, 1996). The pituitary gland lies in the hypophyseal fossa of the sphenoid and is connected to the hypothalamus by the infundibulum, or pituitary stalk. The dura covering the superior aspect of the gland forms the diaphragma sella. The major divisions of the gland are the anterior lobe (adenohypophysis) and the posterior lobe (neurohypophysis). Each of these major subdivisions, which have different

embryological origins, is further subdivided: Adenohypophysis (pars distalis, pars intermedia, and pars tuberalis); Neurohypophysis (pars nervosa, pituitary stalk, median eminence). The anterior lobe is an upgrowth of ectoderm from the roof of the stodeum, while the posterior lobe is a downgrowth of neuroectoderm from the diencephalons. Rathke's pouch grows upwards from the roof of what will become the mouth towards the developing brain. As the upgrowth contacts a downgrowth from the brain, the infundibulum, it begins to pinch off from its connection with the stomodeum. The connection between Rathke's pouch and the oral cavity degenerates and the cells of Rathke's pouch proliferate to form the pars distalis, and extend up the anterior aspect of the infundibulum as the pars tuberallis (Bielańska-Osuchowaska 1975). The posterior surface of Rathke's pouch does not proliferate but forms the poorly developed pars intermedia (Wingstrand et al., 1951; Conklin 1962). Both the adenohypophysis and the neurohypophysis become apparent in porcine embryos with a crown rump length of 10 mm approximately at 20 days of gestation (Patten, 1948).

2. Posterior pituitary (Neurohypophysis)

The posterior lobe or neurohypophysis is a continuation from the hypothalamus: it contains axon terminals that arise from large cell bodies within the supraoptic and paraventricular nuclei of the hypothalamus (Samson et al., 1996). The axons of these neurons run down the pituitary stalk to terminate on the capillary bed of the posterior lobe. These axon terminals store and secret neurohormones (oxytocin and vasopressin) rather than neurotransmitter. So they end near capillaries. The axons of the hormone producing cells can be seen to contain numerous small clear vesicles, and larger dense core vesicles.

3. Anterior pituitary

The anterior pituitary consists of three regions (pars distalis, pars intermedia, and pars tuberalis) within the sella turcica and lies outside the blood-brain barrier. It does not contain nerve terminals and forms of a range of cells producing several hormones. An essential component in the organization and function of the anterior pituitary is the close relationship between the cells and the capillary bed. The access to the circulation promotes uptake of the substrate for hormone synthesis and allows efficient release of hormones into the systemic circulation. The activity of the cells of the anterior pituitary is controlled through the presence of releasing and inhibiting factors in the capillary blood. It has been known that there are colloid droplets within the hypophysial sinusoid considered to be visible aspects of grandular secretion. These intravesicular droplets were observed in great abundance particularly in young and pregnant women (Romeis, 1940; Herlant et al., 1953). The releasing factors are formed by hypothalamic cells and released into the hypophyseal portal circulation.

There are five distinct types of endocrine cell, distributed either throughout the anterior pituitary or localized to particular parts. These topographical localizations have well established the functional significance of different cellular forms (Herlant, 1964; Sétáló et al., 1972, 1976). In the median region of the anterior lobe, cells containing glycoproteinaceous vesicles are predominant (Baker, 1974). Somatotrophs (GH cells) are found generally in the lateral extensions of the lobe, secreting growth hormone. Corticotrophs are located mainly in the center of the lobe, secreting adrenocoticotrophic hormone, beta-lipotrophin, alpha-melanocyte stimulating hormone and beta-endorphin. Thyrotrophs are concentrated mainly in the anterior of the lobe, secreting thyroid-stimulating hormone (TSH). Lactotrophs are

scattered throughout the lobe, secreting prolactin (PRL). Gonadotrophs also scattered throughout the lobe, secreting follicle stimulating hormone (FSH) and luteinizing hormone (LH). However, this is not a general rule and cellular cords of the anterior lobe often have a heterogeneous composition.

Early histological methods were based on the affinity for acidic and basic dyes. This resulted in cells being described as acidophils, basophils or chromophobes that did not take up dye: α-cells (somatotrophs) corresponded to the acidophils while β-cells (follicle stimulating hormone cells) corresponded to the basophils. They are further distinguished as cyanophile cells (δ-cells), orangophile cells (ϵ-cells), and pregnancy cells (η-cells). Modern methods of identification are based on immunohistochemical identification of hormones and by the ultrastructural appearance of the cells.

Experimental investigations have used cytological, ultrastructural, immunocytochmical, and radio immuno-assay (RIA) methods to follow common and species-specific properties of neuroendocrine cells and the nature of their reactions to different stimuli, the similarity and specificity of cell-type reactions to stress, and their role in regulating growth or reproduction. On the basis of the observation that the amino acid sequences of pituitary hormones have a certain common overlapping biological activity and specificity (Li et al., 1972), and that the pituitary cells are genetically programmed and differentiated from chromophobes, there are three proposed cell types: (1) ACTH (corticotropin) and MSH (melanocyte-stimulating hormone) cells, (2) PRL and GH cells, and (3) glycoprotein hormone-producing cells (Pantic et al., 1975, 1990). The specific pituitary cells are synthesizers of hormone and from these cells corresponding families of peptidic or glycoprotein hormone are released. As a source of these hormones, these cells interact with the neighboring cells. They are target cells for

peptides, steroids, and bioamines, and they also have receptors for molecules present in the intercellular matrix. As methodological approaches have advanced, cytological and molecular biological criteria have been used different pituitary cell types characterized. The main parameters are the cell size and shape and the nature of nuclear and cytoplasmic organelles.

The ultrastructure and immunocytochemistry, the nature of granules and amount of hormones, and synthetic and secretory capacities in all of these cell types have been studied under various experimental conditions (Pantic, 1974; Denf et al., 1984). The light and electron microscopic properties of the pituitary cells of pig suggested the extension of Rathke's pouch from the stomodeal ectoderm to the base of the infundibulum, throughout embryogenesis (Nelson, 1933). In view of the frequency of close apposition between gonadotropic and prolactin cells, ACTH and GH and the other cells, communication between neighboring cells is important, not only for inductive interactions and pathways of specific differentiation, but also for their role in regulation of their synthetic and secretory capacity.

The anterior pituitary, as an endocrine gland, is composed of different cell types, each producing different peptide hormones (Herlant, 1964). Adrenocorticotropic hormone, PRL, GH, TSH (thyrotropin), and GTH (gonadotropin)are secreted from separate anterior pituitary cells (Herlant, 1964; Farquhar, 1971; Dacheux, 1984). Nakane (1970) suggested that FSH and LH are produced by a single type of cell. However, in addition to these hormones being produced by specific pituitary cells, these cells also synthesize and release other hormones and molecules into the intercellular matrix by paracrine and endocrine secretion. These molecules have an extremely important role in the regulation of neighboring pituitary cells, their differentiation, activities, behavior, aging, senescence, or degeneration (Schwartz,

2000). Anterior pituitary cells producing PRL, GH, GTH, and TSH were separated mainly according to size and density (Denef et al., 1982).

Exocytotic membrane fusion pore in live cells

Enzyme secretory cells, nerve cells, and endocrine cells are highly specialized cells that release their chemical for intercellular communication by exocytosis. In regulated exocytosis, membrane bounded secretory vesicles are transported, dock, and fuse to specific sites at the plasma membrane to release their contents in response to a physiological stimulus. Earlier electrophysiological studies on master cells (Alvarez et al., 1993; Monck et al., 1995) and adrenal chromaffin cells (Jamieson et al., 1972) suggest the existence of "fusion pores" that irreversibly expand (total fusion) or close (total fusion) after stimulation of secretion. Taking the great advance of high resolution power (at best 300-400 nm), atomic force microscopy (AFM) reveal exocytotic plasma membrane structure, fusion pore, [POROSOME] and its dynamics in both exocrine and neuroendocrine cells (Prekereis et al., 1997; Schneider et al., 1997; Cho et al., 2002a). This discovery demanded reconsideration on the commonly accepted final step in exocytosis, total fusion that total incorporation of secretory vesicle membrane at the cell plasma membrane followed by compensatory retrieval of excess membrane by endocytosis at a later time.

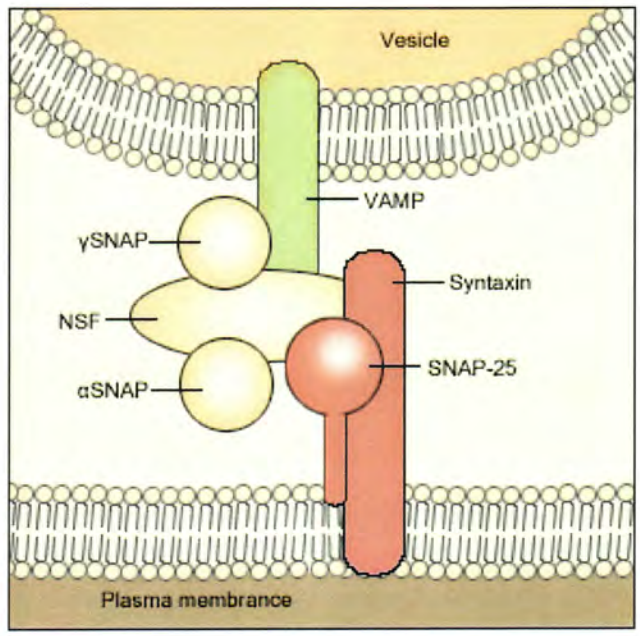
In AFM studies on live acinar cells, a group of circular "pits" measuring 0.5-2 μm and containing depression measuring 100-180 nm in diameter were identified at the apical plasma membrane of resting cells. Following the stimulation of secretion, however, each cone-shaped depression dilated with a 35 % increase in diameter and 25-50 % increase in the depth, returning to resting size after completion of secretion (Schneider et al., 1997; Cho et al., 2002

b). High resolution AFM images in GH secreting cells of the anterior pituitary gland and chromaffin cells (Cho et al., 2002a, b) also revealed the presence of pit and depression structures on their plasma membrane. In resting GH cells, depressions measure 154 ± 4.5 nm (mean ± SE) in diameter. Stimulated GH cells exposed to GH secretagogue (GHS) caused 40 % increase in depression diameter (215 ± 4.6 nm; $P < 0.01$) without change in pit size.

AFM studies using amylase-specific immunogold AFM studies showed localization of amylase at depressions after simulation of secretion, which confirms that depression is the fusion pore in pancreatic acinar cells (Cho et al., 2002b). In GH cells of the pituitary, gold-tagged GH-specific antibodies were localized at depressions following stimulation of GH secretion (Cho et al., 2002a).

A set of proteins involved in the docking and fusion in the two neuronal secretory organelles (synaptic vesicles and large dense-cored vesicles) has been identified, underlying exocytosis (Figure 1). Some of these synaptic proteins such as SNAP-25 and syntaxin are located at the plasma membrane, whereas others like synaptotagmin, synaptobrevin, and synapsin are located on synaptic and large dense-cored vesicle membranes (Linial and Parnas, 1996). Additionally, N-ethylmaleimide-sensitive fusion protein (NSF) and NSF attachment protein (SNAPs) are essential for membrane trafficking (Rothman, 1994). Interactions between plasma membrane proteins (t-SNAREs: SNAP-25 and syntaxin) and vesicular membrane proteins (v-SNARE: synaptobrevin) with soluble factors (NSF and SNAPs) are also critical for interaction of the fusion process (Söllner et al., 1993; Rothman, 1994). Therefore it is acceptable that plasma membrane-associated t-SNARE is a part of the fusion pore complex.

Figure 1. Schematic diagram depicts docking of synaptic vesicles involving the formation of a complex that includes proteins from the cytoplasm: synaptobrevin (VAMP), N-ethylmaleimide-sensitive fusion protein (NSF), NSF attachment protein (SNAP).



CHAPTER 1. IMMUNOCYTOCHEMICAL STUDY ON THE DISTRIBUTION PATTERN OF GROWTH HORMONE CELLS IN THE PORCINE ANTERIOR PITUITARY

A paper prepared as an abstract for the annual meeting of Endocrine Society, Philadelphia, June 2003

Jin-Sook Lee, Srdija Jeftinija, Ksenija Jeftinija, Marvin H. Stromer, and Lloyd L. Anderson

ABSTRACT

The anterior pituitary gland contains cells that produce and secrete growth hormone (GH) into the circulating blood that plays an important role in determining body composition to maintain a beneficial ratio between skeletal muscle and fat. Morphological classification and localization of the pituitary endocrine cell types including GH cells (somatotrophs) have been well established. However, spatial distribution patterns of GH cells in the gland in animals of different ages were not fully described although its characterization may be considered a reflection of histo/cytogenesis for normal secretory function. The objective of our immunohistochemical study was to identify the spatial distribution patterns of GH secreting cells of the newborn and prepubertal porcine pituitary. We designated regions (1, 2, 3, 4, 5), positions (a, b, c), and depth (proximal, middle, and distal) on the 10 μm thick sections for consistent counting; regions 1, 5 (lateral wing of the anterior lobe), regions 2, 4 (shoulder areas), region 3 (center); position a (anterior part; nearest intermediate lobe), position b (middle), position c (exterior); depth-proximal (nearest brain or pituitary stalk), middle (largest part of the gland), and distal (bottom part of gland). Immunoreactive GH cells were round in shape and ranged from 10 to 20 μm in diameter in 1, 42, and 100 day-old pigs. However, a distinctive pattern was found in the distribution of GH cells throughout the

gland over these age groups. Characteristics of this pattern included a high population of GH cells (43.8 ± 1.2 per 30,495 μm^2, mean ± SEM) in regions 1, 5 from proximal to distal and a relatively low population (21.8 ± 1.4 per 30,495 μm^2) of cells in regions 2, 4 from proximal to distal ($P < 0.05$). No significant differences were found among the total GH cell counts per 30,495 μm^2 across the three age groups. However, there was a significant increase of GH cells in region 3 from proximal to distal (20.1 and 36.4 per 30,495 μm^2, 55.2 %>; $P < 0.05$) in all age groups and a decreasing population of GH cells in region 3 between days 1, 42, and 100 (30.0 and 10.0 per 30,495 μm^2, 33.3 %<; $P < 0.05$). Different immunoreactive density of GH cells across the age may reflect changes in the number of GH vesicles or heterogeneity of GH cells. Our observations suggest that there may be regional specificity of cellular differentiation and transformation to facilitate GH secretion in the need for endocrine regulation as the animal ages.

INTRODUCTION

Growth hormone (GH) is a protein hormone composed of 190 amino acids with a molecular mass of 22 kd (Abdel-Meguid et al., 1987; Kato et al., 1990). GH has been recognized as a primary regulator that plays an important role in determining body composition to maintain beneficial ratio between skeletal muscle and fat. Animals deficient in GH lack normal skeletal and muscular development (Ford and Anderson, 1967). Fetuses at 40 days have measurable concentrations of GH, and GH increases 40-fold to apical values at about 90 days of gestation and its secretion is episodic (Klindt and Stone, 1984; Bauer and Parvizi, 1996). Immediately after birth, serum GH concentrations decline abruptly, followed by elevated secretion at 3 to 5 weeks after birth. These postnatal rises in serum GH secretion

occur before weaning, thus dietary changes are not the cause of this increase (Owenes et al., 1991; Buonomo and Klindt, 1993). The pattern of change in serum GH concentrations associated with pubertal development mimics the developmental pattern in contemporary breeds of boars, albeit sooner (Louveau et al., 1991; Lunstra et al., 1992).

GH secretion results from a complex series of interactions that occur both in peripheral tissue and in the central nervous system (CNS). A role for GH in the brain is more critically approached because its pulsatile secretion is regulated by a dual system of hypothalamic control: a positive/negative feedback loop. Growth hormone-releasing hormone (GHRH) is released from arcuate neurons in the hypothalamus and transported through the portal blood vessels into the pituitary gland, where it stimulates GH release from somatotrophs (GH-secreting cells in the anterior pituitary gland). Negative feedback is mediated by the release of somatostatin (SS) from hypothalamic neurons that act to inhibit GH release from somatotrophs in the anterior pituitary (Guillemin et al., 1982; Frohman et al., 1992; Bertherat et al., 1995).

Numerous immnunohistochemical investigations have helped to identify the morphological classification and distribution of the anterior pituitary cell types including somatotrophs in rat (Setalo et al., 1976; Watanabe et al., 1979; Sasaki et al., 1988), hamster (Thompson et al., 1976), human (Pelletier et al., 1978; Monique et al., 1985; Asa et al., 1988) and pig (Nelson et al., 1933; Bielanska et al., 1975; Liwska et al., 1978; Dacheux, 1984). Heterogeneity of the cell distribution pattern in the anterior pituitary may be considered a reflection of histo/cytogenesis. The localization of the anterior pituitary cells occurs during fetal period in human (Conklin, 1968), rat (Watanabe and Daikoku, 1979), mouse (Japon et al., 1994), and pig (Sasaki et al., 1992). Recent studies in molecular biology have

demonstrated the timing of the appearance of several nuclear transcription factors that regulate the differentiation of the anterior pituitary cells and the onset of hormone production in fetal mice (Wakins and Camper, 1998). There are changes in level of GH and anterior pituitary cells at different reproductive stage in pig. The earlier study (Anderson et al., 1972) showed that the proportion of GH cells was higher in 17-day-old pigs than in any reproductive stages in mature animals because of the rapid growth in the young pig. The finding in the young pig of the highest percentage of GH cells correlated with the extraordinarily high basal plasma growth hormone level (50 to 200 ng/ml) in newborn and 21-day-old pigs (Kipnis et al., 1969). Although there have been a numerous studies on regulation of GH secretion from the anterior pituitary regarding the relationship to other hypothalamic/pituitary hormones, only several morphological, immunohistochemical, and immunoelectron microscopic studies of the GH cells in the porcine anterior pituitary have been reported (Dacheux 1980, and Sasaki, 1992). The aim of the current study is to detect immuno-reactive GH secreting cells in the newborn and prepubertal porcine pituitary using an advanced immunohistochemical technique and identify spatial distribution patterns of the GH secreting cells in the anterior pituitary, of which little attention has been paid.

MATERIALS AND METHODS

Experimental Animals

Yorkshire pigs, raised at the Iowa State University Animal Nutrition Farm, were used for these experiments. Day 1, day 42, and day 100-old pigs were euthanized by electrocution and decapitated. Three pigs from each age group were selected for this study. Animal care

and experimental protocols were in accordance with the guideline and approval of the Iowa Sate University Committee on Animal Care.

Preparation of pituitary glands

Whole pituitary glands were immediately removed from the pig brains and fixed in cold 4% paraformaldehyde for 2 days and transferred into cold 30% sucrose until they sank to the bottom. Tissue was embedded in Tissue-Tek O.C.T. compound (Sigma-Aldrich, St. Louis, MO) for cryostat sectioning (Jung Frigocut 2800N, Leica Instruments, TX). Coronal sections (10 μm thick) of a whole pituitary gland were cut from proximal to distal end, sequenced serially. The number of sections ranged from 216 to 378). All sections were placed on poly-L-lysine (0.05 mg/ml, MW 100,000; Sigma) coated slides, allowed to dry at room temperature for 24 h, and stored in a freezer (-20°C) until use for immunohistochemistry.

Immunohistochemistry

Serial sections were stained by the immunoperoxidase/avidin-biotin system to identify immunoreactive GH cells in the anterior pituitary. Tissue sections were first washed three times in 50 mM potassium phosphate buffered saline (KPBS) and then treated with 0.3% hydrogen peroxide in KPBS for 30 min to neutralize endogenous peroxidase activity. The sections were incubated for 1 h at room temperature with 0.7% normal goat serum in KPBS containing 1% bovine serum albumin (BSA) to block nonspecific binding and 0.4% triton X-100 (MW 628; Fisher Biotech, Fair Lawn, NJ) to permeabilize the membrane. The slides were then incubated overnight with monkey anti-porcine GH (pGH) antibody (1:500,000

dilution for day 1 and day 42 pigs, 1:100,000 dilution for day 100 pigs in 50 mM KPBS containing 1% BSA; gift from Dr. A. F. Parlow, National Hormone & Pituitary Program, Harbor-UCLA Medical CTR, Torrance, CA) at room temperature in a humidified chamber. The sections were then thoroughly washed and treated for 1 h with biotinylated goat anti-human IgG (1:500 dilution), rewashed, and treated with avidin-peroxidase compound (Vector ABC-Elite kit;Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After washing, peroxidase activity was carried out using 0.04% 3,3'-diaminobenzidine tetra hydrochloride (FW360.1; Sigma) containing nickel sulfate (2.5%) and 0.1% hydrogen peroxide in 0.1 M sodium acetate for 6 min to visualize immunoreactive GH cells as a black colored reaction product. Subsequently, tissue sections were dehydrated in 70%, 85%, 95%, and 100% ethanol each for 3 min and cleared in xylene for 3 min. The slides were coverslipped using acrytol (Surgipath Medical Industries, Graylake, IL) as mounting medium.

Controls

Negative control immunohistochemical tests were carried out by substituting normal goat serum or 50 mM KPBS for the primary antisera on some sections of the serial sets. No staining was observed in any negative control section.

Quantitative analysis

Ten sections of each serial set were preliminarily examined under the lowest magnification (10× objective) to confirm that the change of cell distribution pattern is consistent from proximal to distal. Then, three sections per pituitary gland were selected in

order from proximal (near to the brain or pituitary stalk), middle (largest part of gland), and distal (bottom); depicted in Figure 1. Anterior lobes containing somatotrophs were divided into 5 regions (region 1, 2, 3, 4, 5; regions 1, 5-lateral wings of anterior lobe, regions 2, 4-shoulder areas, region 3-center) under a 10× objective (final magnification 125×). Three different positions (position a, b, c; position a-anterior part nearest to intermediate lobe, position b-middle, position c-exterior) of each micrograph (unit area 30,495 μm^2) along the regions were taken under a 40× objective (final magnification 500×) for quantification (Figure 2). This designation of dividing regions and positions for counting was reasonable to identify the distribution pattern of immunoreactive GH cells because each pattern was sketched for the whole image of the section. The fifteen positions along the five regions were quantified in each section selected from three serial depths of gland. The number of GH cells in each region/position/depth of the same age group was averaged.

Statistical analysis

The General Linear Model of the Statistical Analysis System was used to evaluate multi variance (age, depth, region, position) effects. Statistical analyses of the counts of the GH cells at different region/position/depth were determined by the Student's t-test. The number of GH cell count in each region/position at each depth represent the mean of the same age group (n=3 each day 1, day 42, and day 100).

RESULTS

Light microscopic observations

Immunoreactive GH cells in the anterior pituitary were round in shape and ranged from 10 to 20 μm in diameter in 1, 42, and 100 day-old pigs. There was a difference in imunoreactive density of the positive cells; densely stained (black color)/slightly stained (gray color) GH cells were appeared. No significant difference among the total GH cell counts per 30,495 μm^2 across the three age groups was observed. Three sizes of population of GH cells per 30,495 μm^2 were identified as high, medium, and low population: > 40, 20 to 40, < 20 (Figure 3). The pattern of GH cells in the anterior lobe was distributed bilaterally symmetrical.

Spatial distribution pattern of GH cells

Day 1 (Figure 4a, b, c)

Significantly high number of GH cells in regions 1, 5, and 3 from proximal to distal compared to the cells in region 2, 4 was found ($P < 0.05$). Based on this feature, general pattern from proximal to distal was W-shape. Particularly, the pattern of position c at region 3 had a significant increase in number of GH cells at distal ($P < 0.001$).

Day 42 (Figure 5a, b, c)

High number of GH cells was maintained in region 1, 5 compared to region 2, 4 ($P < 0.05$). There was a distinctive difference in distribution pattern in region 3 from that in day 1. Proximal had a decreasing population of GH cells at region 3-b, c in day 42 compare to the one in day 1 (67 and 43.7 per 30,495 μm^2, 34% <; $P < 0.05$). However, the pattern at region

3 also appeared flip-over shape at distal because of a significant increase of GH cell number ($P< 0.001$).

Day 100 (Figure 6a, b, c)

High number of GH cells was found in region 1, 5 only at proximal and the pattern at this depth levels was bell (U) shape. There was a significant decreasing number of GH cells at region 3-a, b, c compare to the one in day 42 (81 and 56.3 per 30,495 μm^2, 30.5%<; $P < 0.05$). Because region 3-a, b, c had a significant increase in GH cell number at distal, immunoreactive GH cells were distributed widely all over the section.

General characteristics of the distribution pattern (Figure 7)

GH cells were densely distributed in region 1, 5 from proximal to distal (43.8 ± 1.2 per 30,495 μm^2, Mean ± SEM), whereas rarely distributed in region 2, 4 from proximal to distal (21.8 ± 1.4 per 30,495 μm^2, Mean ± SEM) in day 1, day 42, and day 100. However a significant increase of GH cells in region 3 at distal was observed in all age groups (55.2%>, $P < 0.05$). This characteristic showed the flip-over shape in number of GH cells at distal. There was a trend of decreasing population of GH cells in region 3 between day 1, day 42, and day 100 (23.6% & 30.5%, total 54.1%; at $P < 0.05$). Therefore, the pattern across the age was changed from W shape to Bell (U) shape; high number of GH cells in region 1, 3, 5 was maintained in day 1 and gradual decreasing GH cell number in region 3 particularly at proximal (Figure 8). A remarkable change in region 3 in the spatial pattern across the depth reveals the highest GH cell population at the distal (Figure 9).

DISCUSSION

This study is the first attempt to investigate the spatial distribution pattern of GH cells in the newborn and prepubertal porcine anterior pituitary with fine details of counting regions, using the advanced immunohistochemical technique. The morphological classification and distribution of the anterior pituitary cell types including somatotrophs have been identified in rat (Setalo et al., 1976; Watanabe et al., 1979; Sasaki et al., 1988), hamster (Thompson et al., 1976), human (Pelletier et al., 1978; Monique et al., 1985; Asa et al., 1988) and pig (Nelson et al., 1933; Bielanska et al. 1975; Liwska et al., 1978; Dacheux, 1984). It is necessary to consider anterior pituitary ontogeny in order to understand the reason for the cellular localization. In the fetal rat pituitary, the first GH-immunoreactive cells were found during late pregnancy: at 18 days (Watanabe and Daikoku, 1979) and 19 days of gestation (Setalo and Nakane, 1972, 1976; Chatelain et al., 1979; Hemming et al., 1986). In the fetal pig pituitary, however, the first immunoreactive GH cells were detected at 45 days of gestation (Danchin and Dubois, 1982) and from 40 to 50 days of gestation (Dacheux, 1984). The plasma GH concentrations in fetal pigs increased at 70 days of gestation to become about 10 to 15 × the level in the dam (Antinmo et al., 1976). The earlier study on GH cell distribution patterns in fetal pig pituitary (Sasaki et al., 1992) showed that GH cells were densely distributed in all areas especially except in large parts of the rostral area in almost all areas in lateral wings in the sagittal section of the gland after 60 days of gestation. Conklin (1968) reported that the different segments facing Rathke's pouch might contain the potential differences to produce various types of anterior pituitary cells and defined the order and the portions in which each type of chromophil appeared. The LM study (Sasaki et al., 1992) revealed that GH and PRL cells in the fetal pig anterior pituitary had complementary cellular

density. Porter et al. (1991) proposed the transformation of somatotrophs (GH cells) to mammotrophs (prolactin cells) via somatomammotrophs (both GH and PRL secreting cells) in the lactating rats. The light microscopy study on somatomammotrophs in the female goat anterior pituitary (Shotaro et al., 2001) suggests that somatomammotrophs might increase by cessation of lactation and it may be caused from a transformation of a part of mammotrophs. In our study, the region 3-a, b (the regions originated from embryonic Rathke's pouch) had a high population of GH cells in day 1, but remarkable decreasing number of cells as age gets old, which suggest that region 3 may be involved for the cellular differentiation and transformation in need of endocrine regulation after birth until the prepubertal age.

The ultrastructural immunocytochemical study (Dacheux, 1984) on porcine anterior pituitary cells demonstrate that increase in number and size of the immunoreactive GH cells including an accumulation of numerous secretory granules slightly larger in diameter is in agreement with the increase in GH-concentrations in blood plasma between day 70 and 105 of gestation, from day 40 to day 90. From our findings in light microscopy observations, however, the number of GH cells per unit μm^2 and the range of immunopositive GH cells across day 1, day 42, and day 100 old animal groups have no significant differences ($P >$ 0.001), which implicate increasing GH level in the young pig is caused by increase not in size but in total number of GH cells as weight or size of the pituitary gland are increased (Table 1). Anderson et al. (1972) also pointed out marked increase in GH during later reproductive stages was caused primarily by increase weight of the pituitary gland.

Immunocytochemical staining of GH cells at different age groups required different optimal concentrations of primary antibody, porcine GH antibody raised in monkey (Table 1). Different immunoreactive density of GH cells across the age may reflect changes in the

number of GH vesicles or heterogeneity of GH cells. Our study suggests that there may be regional specificity of cellular differentiation and transformation to facilitate GH secretion in the need for endocrine regulation during rapid growing period in young pig. The advanced imaging technique for three dimensional reconstruction of distribution pattern with serial sections of large size models is needed to be developed for better visualizing spatial characteristics. Since pituitary is an endocrine gland with a complex and heterogeneous distribution of cells functionally communicating with neighbor endocrine cells, discovering histological changes with other hypothalamic/pituitary hormones could be taken into account in our understanding of pituitary functioning. The characteristics of spatial distribution patterns of specific endocrine cells in pituitary gland may be applied to examine therapeutic strategies for the management of pituitary disorders in both physiological and pathological condition.

REFERENCES

Abdel-Meguid SS, H-S Shieh, Smith WW, Dayringer HE, Violand BN, and Bentle LA 1987 Three-dimensional structure of a genetically engineered variant of porcine growth hormone. Proc. Natl. Acad. Sci. U.S. A.84: 6434-6437

Anderson LL, Peters JB, Melampy RM, Cox DF 1972 Changes in adenohypophysial cells and levels of somatotrophin and prolactin at different reproductive stage in the pig. J. Reprod. Fert. 28:55-65.

Antinmo T, Baldijao C, Pond, W.G., and Barnes, R.H. 1976 Decreased dietary protein of energy intake and developing progeny. J. Nutr. 106:940-946.

Asa SL, Kovacs K, Horarh E, Losinske NE, Laszlo FA, Domokos I, Kalliday WC 1988 Human fetal adenohypophysis. Electron microscopic and ultra structural immunocytochemical analysis. Neuroendocrinology 48:423-431.

Bauer M and Parvisi N 1996 Pulsatile and diurnal secretion of GH and IGF-I in the chronically catheterized pig fetus. J. Endocrinol. 149:125-133.

Bertherat J, Bluet-Pajot MT, Epelbaum J 1995 Neuroendocrine regulation of growth hormone. Euro. J. Endocrinol. 132:12-24.

Bielanska-Osuchowska, Z, and Liwska J 1975 Studies on development of the adenohypophysis in the domestic pig. Folia Porphol. 34:143-149.

Buonomo FC, and Klindt JK 1993 Ontogeny of growth hormone (GH), insulin-like growth factors (IGF-I and IGF-II) and IGF binding protein-2 (IGFBP-2) in genetically lean and obese swine. Domest. Anim. Endocrinol. 10:257-265.

Conklin JL 1968 The development of the human fetal adenohypophysis. Anat. Rec. 160:79-91.

Dacheux F 1980 Ultrastructural immunocytochemical localization of prolactin and growth hormone in the porcine pituitary. Cell Tissue Res. 207:277-286

Dacheux F 1984 Functional differentiation of the anterior pituitary cells in the fetal pig: An ultrastructural immunocytochemical study. Cell Tissue Res. 235:623-633.

Danchin E, and Dubois MP 1982 Immunocytological study of the chronology of pituitary cytogenesis in the functioning of the hypothalamo-pituitary-gonadal axis. Reprod. Nutr. Dev. 22:135-151.

Ford JJ, Anderson LL 1967 Growth in immature hypophysectomized pigs. J. Endocrinol. 37:347 Frohman LA, Downs TR, Chomczynski P 1992 Regulation of growth hormone secretion. Font. Neuroendocrinol. 13:344-405.

Guillemin R, Brazeau P, Bohlem P, Esch F, Ling N, Wehrenberg WB 1982 Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly. Science 218:585-587.

Hemming, FJ, Dubois, MP and Dubois PM 1986 Somatotrophs and lactotrophs in the anterior pituitary of fetal and neonatal rats. Cell Tissue Res. 245:457-460.

Japon MA, Rubinstein M, Low MJ 1984 In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J Histochem Cytochem. 42:1117-1125.

Kato Y, Shimokawa, Kato N, Hirai T, Yoshihama K, Kawai H, Hattori M, Ezashi T, Shimokawa Y, and Wakabayashi K 1990 Porcine growth hormone: Molecular cloning of cDNA and expression in bacterial and mammalian cells. Biochem. Biophys. Acta 1048:290-293.

Kipnis DM, Hertelendy F, Machlin L J (1969) Studies of growth hormone secretion. Proc. 3rd int. Congr. Endocrinology. Mexico, D. F., 1968. Excerpta med. Int. congr. Ser. 184: 601.

Klindt J, and Stone RT 1984 Porcine growth hormone and prolactin: Concentrations in the fetus and secretory patterns in the growing pig. Growth 48:1-15.

Liwska J 1978 Ultrastructure of the adenohypophysis in the domestic pig (*Sus scrofa dometica*). Par I: Cells of the pars anterior. Folia Histochem Cytochem. 16:307-314.

Louveau I, Bonneau M, and Salter DM 1991 Age related changes in plasma porcine growth hormone (GH) profiles and insulin-like growth factor-I (IGF-I) concentrations in Large White and Meishan pigs. Reprod. Nutr. Dev. 31:205-216

Lunstra DD, Borg KE, and Klindt J 1992. Characterization of pubertal development in the Meishan Chinese boar. J. Anim. Sci. 70(suppl. 1): 267.

Nelson WO 1933 Studies on the anterior hypophysis. I. The development of the hypophysis in the pig (*Sus scrofa*) II. The cytological differentiation in the anterior hypophysis of the fetal pig. Am. J. Anat. 52:307-332.

Owens PC, Campbell RG, Johnson RG, King R, and Ballard FJ 1991 Developmental changed in growth hormone, insulin-like growth factors (IGF-I and IGF-II) and IGF-binding proteins in plasma if young growing pigs. J. Endocrinol. 128:439-447.

Porter TE, Wiles CD, Frawley LS, 1991 Evidence for bidirectional interconversion of mammosomatotrophs and somatotrophs: Rapid reversion of acidophilic cell types to pregestational proportions after weaning. Endocrinology 129:1215-1220.

Sasaki F, Iwama Y 1988 Sex difference in prolactin and growth hormone cells in mouse adenohypophysis: Serological, morphometric, and immunohistochemical studies by light and electron microscopy. Endocrinology 123: 905-912.

Sasaki F, Ichikawa Y, Yamauchi S 1992 immunological analysis in the distribution of cells in the fetal porcine adenohypophysis. Anat. Rec. 233:135-142.

Setalo G, and Nakane PK 1972 Studies on functional differentiation of the cells in fetal anterior pituitary glands of rats with peroxidase-labled antibody method. Anat. Rec. 172:403-404.

Setalo G and Nakane PK 1976 Functional differentiation of the fetal anterior pituitary cells in the rat. Endocrinol. Experiment 10:155-166.

Shotaro Nishimura, Kentaro Ikeda, Kaorn Okano, Takafumi Gotoh, Shoji Tabata, and Hisao Iwamoto 2001 Proportional changes of somatotrophs, mammotrophs and somatomammotrophs induced by cessation of lactation in female goat adenohypophysis. J. Anim. Sci. 72(1): 32-38.

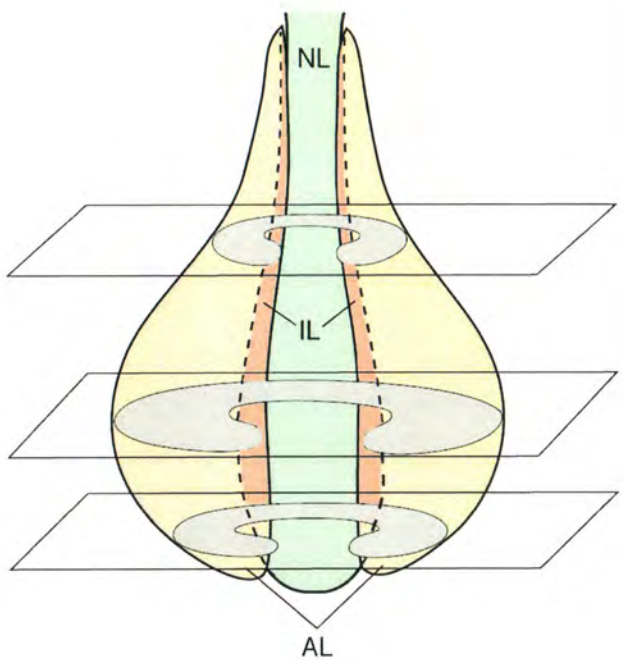
Tompson SA, Trimble JJ 1976 immunohistochemical localization of prolactin cells of the pars distalis in the fetal and neonatal hamster. A light and electron microscopy study. Cell Tissue Res. 168:161-175.

Wakins CD, Camper SA 1998 How many homeobox genes does it take to make a pituitary gland. Trends Genet. 14:384-390.

Watanabe YG, Daikoku S 1979 An immunohistochemical study on the cytogenesis of adenohypophysial cells in fetal rats. Dev. Biol. 68:557-567.

Figure 1. Top view of the pig pituitary gland (left). Sections cut from proximal to distal end of the whole pituitary gland at 10μm thick. NL: Neural Lobe (Posterior Pituitary) IL: Intermediate Lobe AL: Anterior Lobe (Anterior Pituitary); Proximal (nearest brain or pituitary): Middle (largest part of gland): Distal (bottom part of gland)

Experimental Design



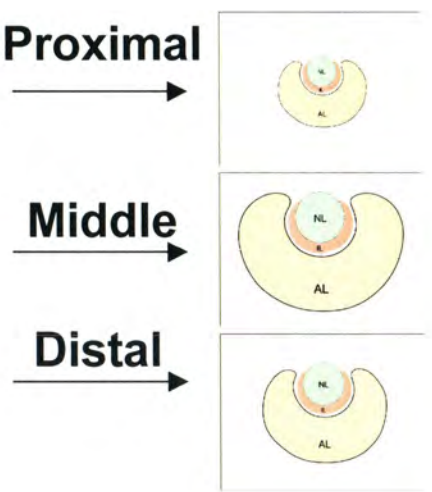


Figure 2. Designation of regions for counting. NL: Neural Lobe (Posterior Pituitary) IL: Intermediate Lobe AL: Anterior Lobe (Anterior Pituitary); regions 1, 5 (lateral wings of the anterior lobe), regions 2, 4 (shoulder areas), region 3 (center); position a (anterior part nearest intermediate lobe), position b (meddle), position c (exterior)

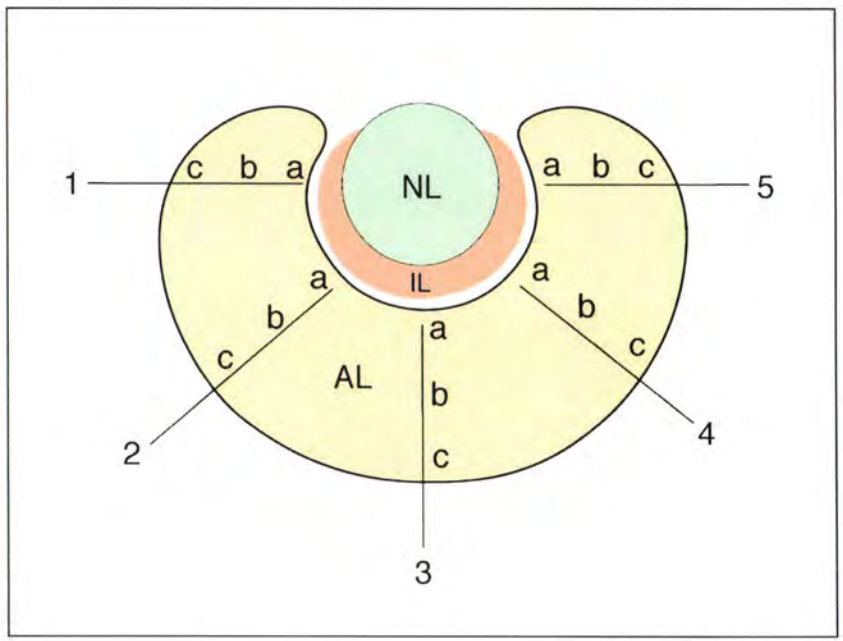


Table 1. Morphological data on pituitary gland and optimal concentration of the primary antibody (Monkey pGH) at different ages in the pigs. (ICC: immunocytochemistry)

Age of the group	No. of pigs	Pituitary gland		ICC staining
		Diameter (mm)†	Fresh weight (mg)†	Optimal concentration of antibody (Monkey pGH)
Day 1	3	4.0 ± 0.25	32.3 ± 1.53	1:500,000
Day 42	3	7.1 ± 0.28	82.0 ± 3.00	1:500,000
Day 100	3	9.0 ± 0.25	252.0 ± 6.24	1:100,000

† Mean of each age-group and standard error are listed

Figure 3. Three sizes of GH cell population in day 1, day 42, and day 100 pig pituitary; **(a)** high (< 20 GH cells per 30,495 μm^2), **(b)** medium (20 to 40 GH cells), **(c)** low (< 40 GH cells). Note cell size ranges from 10 to 20 μm in diameter in all age groups. Final magnification 500X

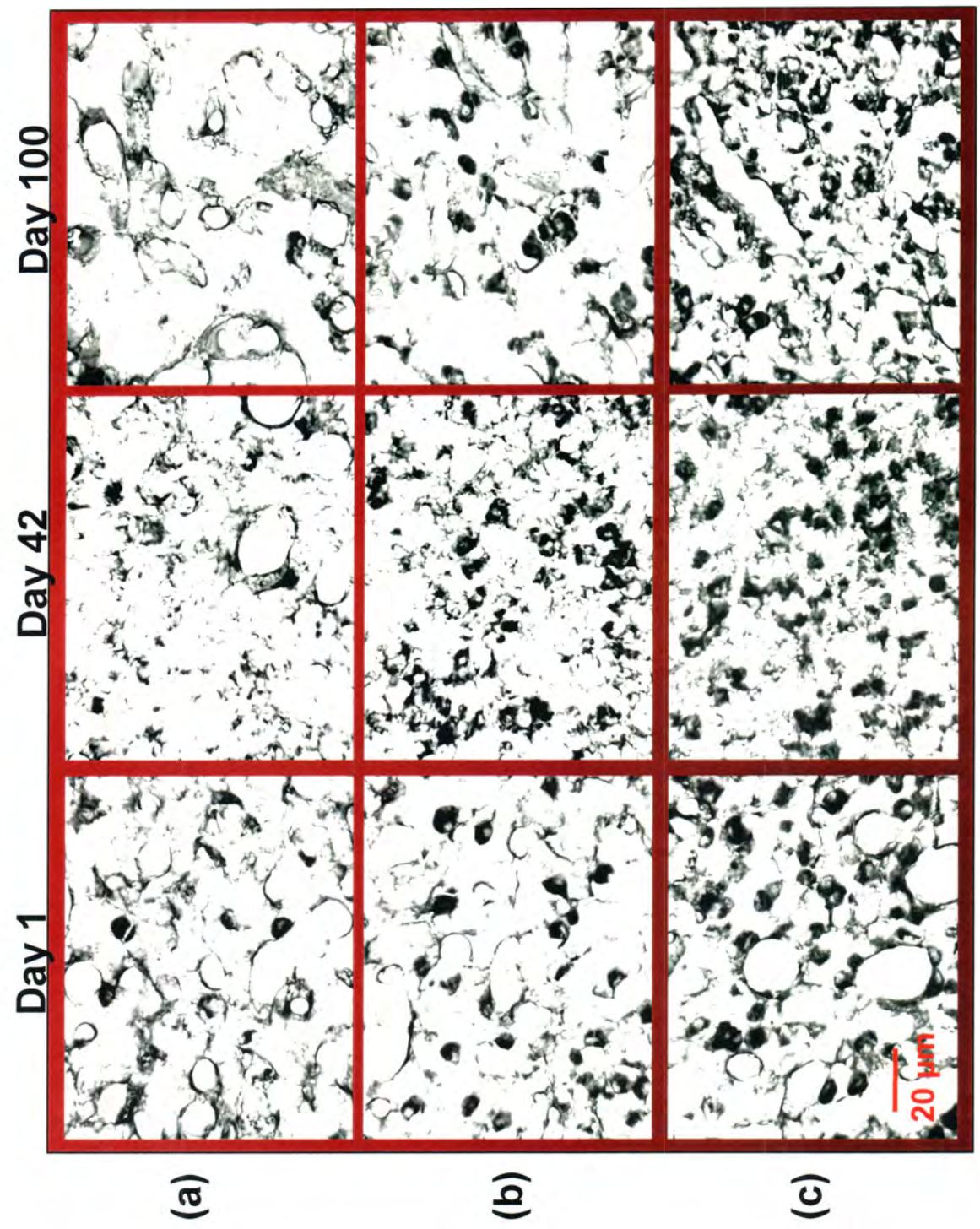

Day 1
Day 42
Day 100
(a)
(b)
(c)
20 μm

Figure 4. Distribution pattern of GH cells in anterior pituitary of day-1 pig. The means of GH counting each region/position were represented in the diagram of the section (left). The density of the GH population was represented by gray colored dot. The distribution patterns of GH cells at proximal (a), middle (b), and distal (c) were represented in the graphs (right).

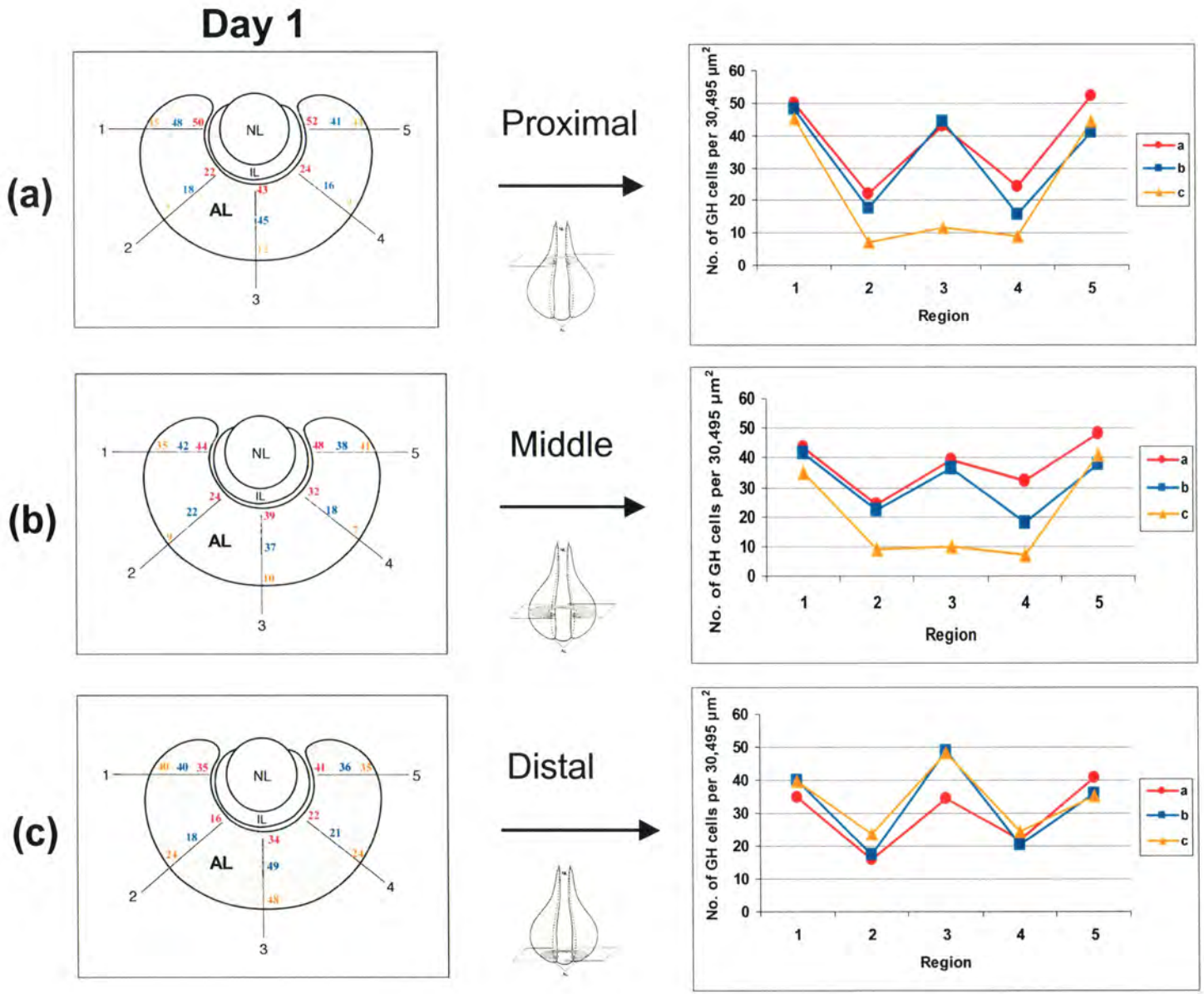
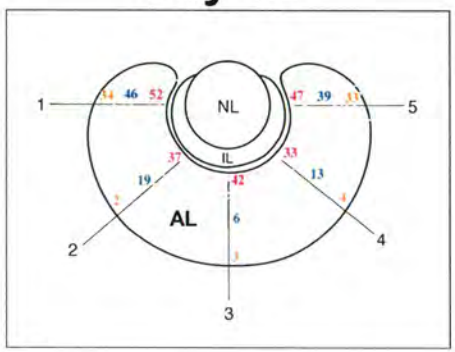

Day 1
(a)
(b)
(c)
NL
IL
AL
1
2
3
4
5
Proximal
Middle
Distal
No. of GH cells per 30,495 µm²
Region
a
b
c

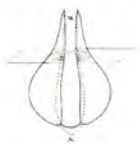
Figure 5. Distribution pattern of GH cells in anterior pituitary of day-42 pig. The means of GH counting each region/position were represented in the diagram of the section (left). The density of the GH population was represented by gray colored dot. The distribution patterns of GH cells at proximal (a), middle (b), and distal (c) were represented in the graphs (right)

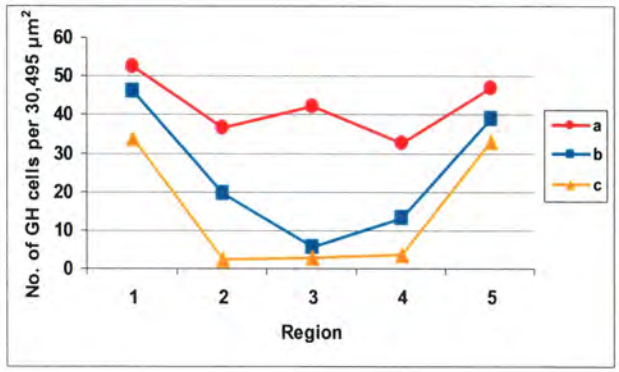
Day 42

(a)

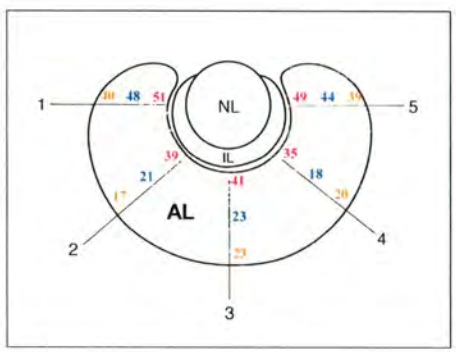


Proximal

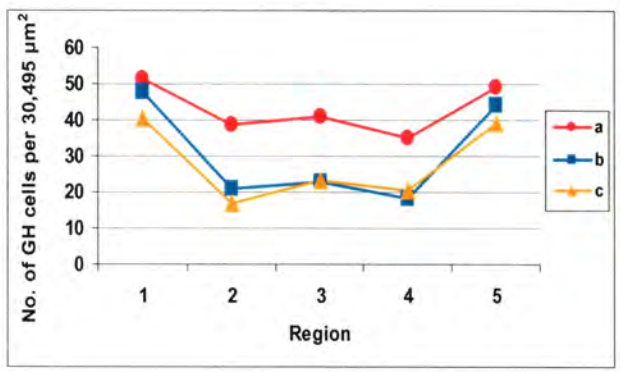




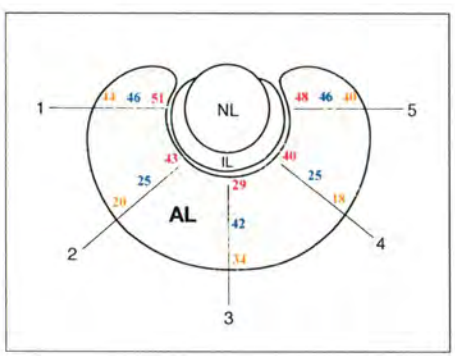
(b)



Middle



(c)



Distal

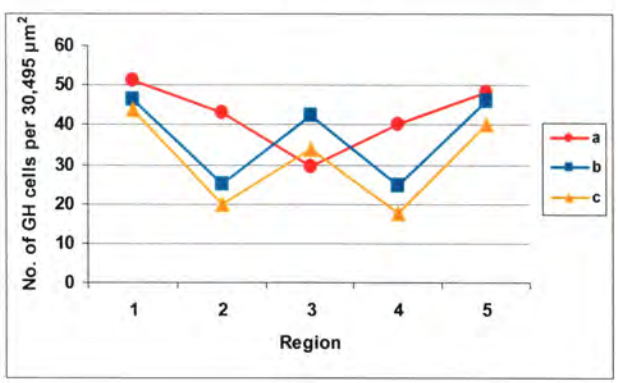
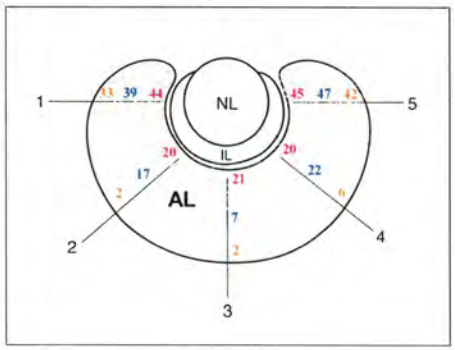


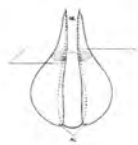
Figure 6. Distribution pattern of GH cells in anterior pituitary of day-100 pig. The means of GH counting each region/position were represented in the diagram of the section (left). The density of the GH population was represented by gray colored dot. The distribution patterns of GH cells at proximal (a), middle (b), and distal (c) were represented in the graphs (right)

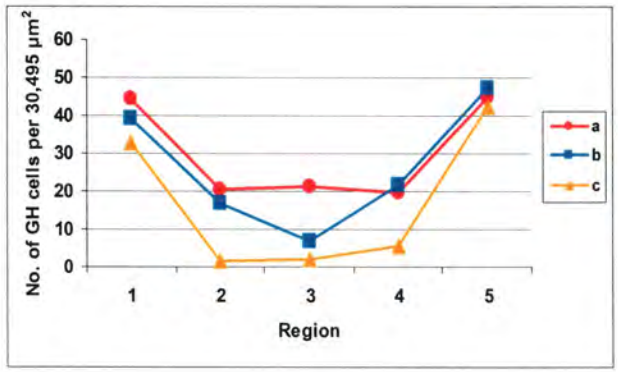
Day 100

(a)

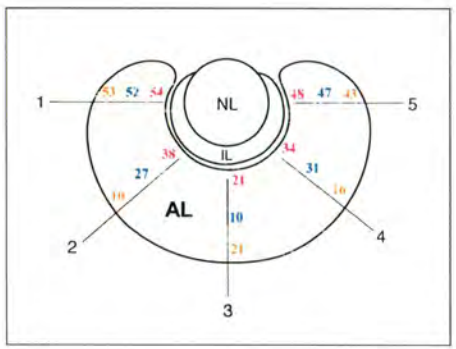


Proximal

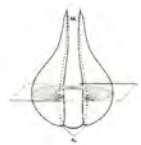


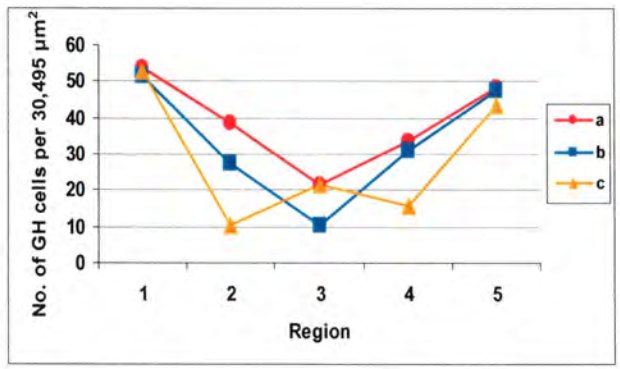


(b)

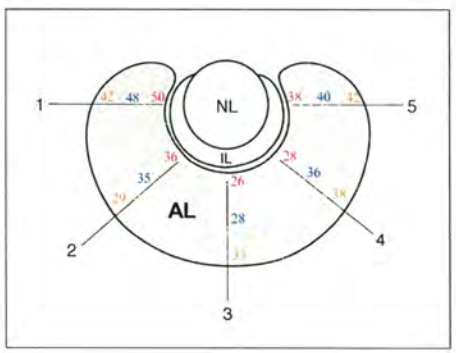


Middle





(c)



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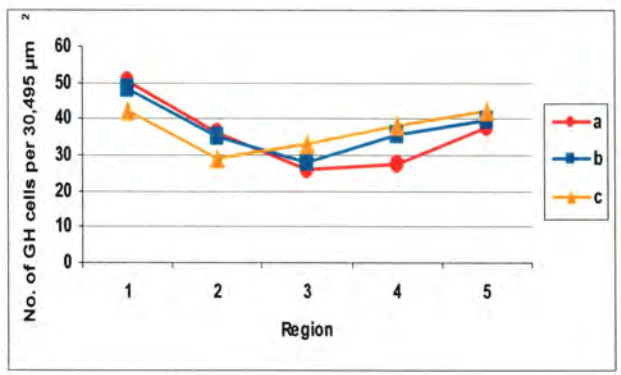


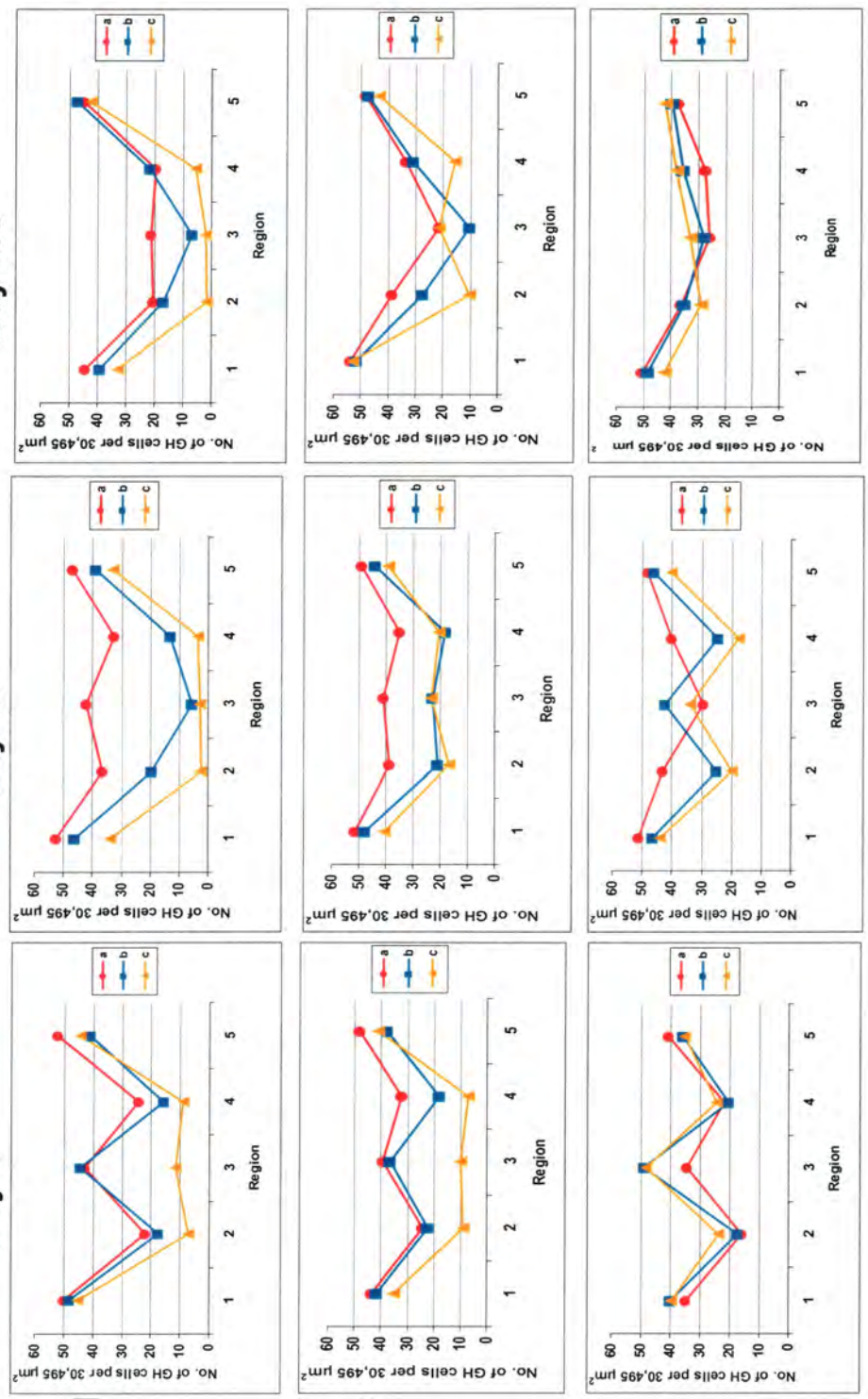


Figure 7. The comparisons of different distribution patterns of GH cells across age groups

Figure 8. Different population of GH cells in region 3-a at proximal with the age: (a) Day 1, (b) Day 42, and (c) Day 100. Area of each image is 30495 μm2 and final magnification is 500X

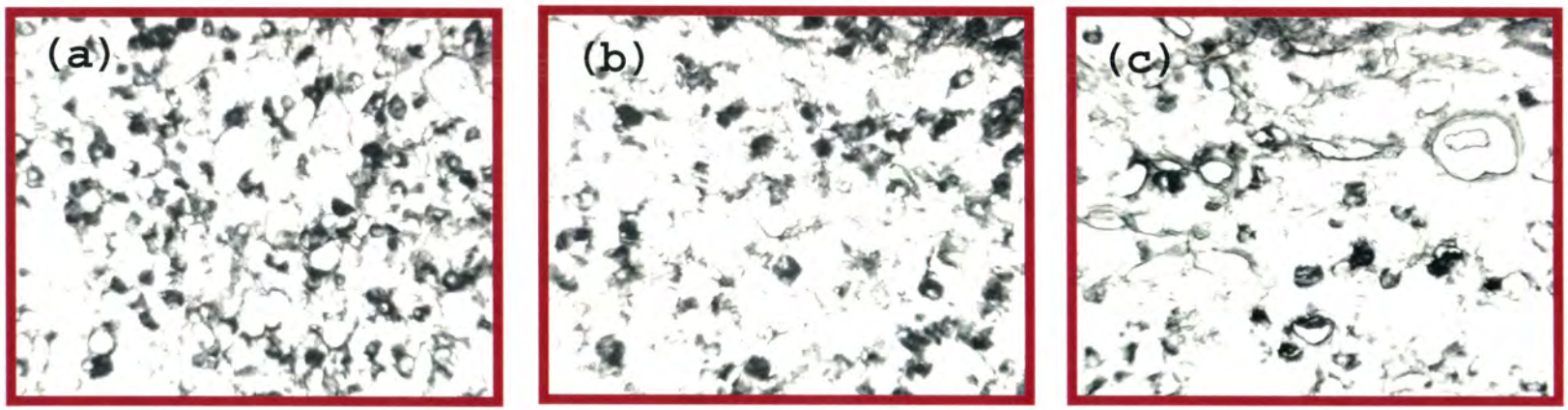
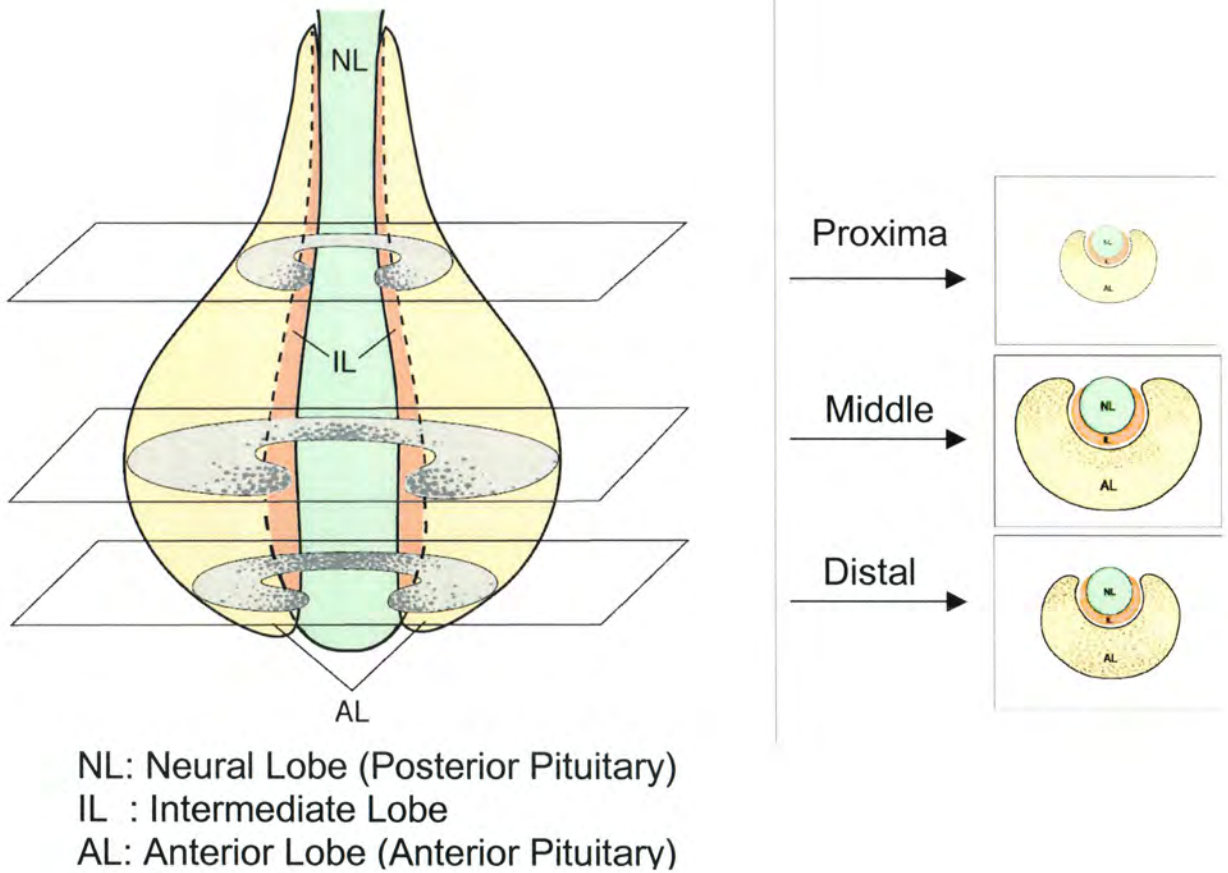


Figure 9. General characteristics of the distribution pattern of GH cells in the porcine anterior pituitary



CHAPTER 2. NUMBER OF SECRETORY VESICLES IN GH CELLS OF THE PITUITARY REMAINS UNCHANGED AFTER SECRETION

A paper prepared as an abstract for the annual meeting of the American Society for Cell Biology, San Francisco, December, 2002

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ABSTRACT

Earlier electron microscopy studies demonstrate that there is no loss of secretory vesicles after exocytosis. However, formation of empty and partly empty vesicles is seen in a variety of cells after discharging of their vesicular content. Transmission electron microscopy (TEM) was used to determine the total number of all categories of secretory vesicles in resting and in GH-stimulated porcine pituitary cells. We identified three categories of vesicles; filled, empty, and partly empty. Each category of vesicle is on a μm^2 of cell area basis, and was determined independently by two persons. Control pituitary cells contained more than twice as many filled vesicles than did the stimulated cells. However, stimulated cells contained nearly twice as many empty vesicles and 2.5 × more partly empty vesicles than did control cells. There was no significant difference in total number of vesicles between control and stimulated pituitary cells. Immunogold labeled secretory vesicles in GH cells revealed that localization of GH is only in electron dense vesicles in control and stimulated cells. Our TEM study on GH cells demonstrates no loss of the number of secretory vesicle following exocytosis. These results extend those from earlier studies on other cell types, consistent with a mechanism that following stimulation vesicles

transiently dock and fuse at the fusion pore to release vesicular contents. The empty vesicles seem to be then recycled.

INTRODUCTION

Exocytosis controlled by external and internal cellular signals is an essential process in enzyme secretion, neurotransmission, and hormone release. Membrane-bounded secretory vesicles are transported to the plasma membrane fusion pore to release of their contents. In the synaptic neurotransmission, vesicles containing neurotransmitter fuse with the presynaptic membrane and discharge their contents into the synaptic cleft. After the process of exocytosis, the vesicular membrane is retrieved by endocytosis for reuse (Betz and Angleson, 1998; Hause and Reese, 1973; Ceccarelli et al., 1973). Studies using transmission electron microscopy on stimulated secretory cells demonstrate the presence of several intact but empty or partly empty secretory vesicles (Lawson et al., 1975). Transmission electron micrograph of rat mast cells reveal the presence of several spent vesicles as empty or partly empty membrane-bounded cavities in continuity with the plasma membrane following exocytosis (Lawson et al., 1975). There is accumulating evidence demonstrating no change in the number of secretory vesicles following stimulation of secretion. Earlier studies demonstrate that, following secretion, there is depletion of vesicular contents, resulting in an increase in empty and partly empty vesicles and no loss of number of secretory vesicle. Quantitative EM on stimulated and resting bovine chromaffin and rat pancreatic acinar cells showed no significant change in the number of peripheral dense-core vesicles after stimulation of secretion (Platter et al., 1997). The study (Cho et al., 2002a, Schneider et al., 1997) in live pancreatic acinar cells using atomic force microscopy (AFM) demonstrates only

a 25-35% dilation of the 100-180 nm in diameter fusion pores following secretion. Parallel study using electron microscopy (Cho et al., 2002b) showed no loss of secretory vesicle number after exocytosis. Their AFM and TEM studies confirm that transient fusion of secretory vesicles at the cell plasma membrane may be the rule in exocytosis. Growth hormone (GH) produced by specialized cells (somatotrophs) of the anterior pituitary gland is stored in secretory vesicles that are synthesized from the Golgi apparatus to the plasma membrane. Cho's study suggests that, after stimulation of secretion with a GH-secretagogue (i.e., L-692,585), the 350-500 nm in diameter membrane-bounded secretory vesicles in GH cells may also dock and transiently fuse with plasma membrane to release vesicular contents showing a 40% increase in the size of 100-200 nm 'depression'. This study was undertaken to determine the fate of secretory vesicles in GH cells of porcine pituitary after stimulation of GH secretion. Our TEM study reveals that unchanged number of secretory vesicles following exocytosis in GH cells demonstrates membrane-bound secretory vesicles transiently dock and fuse to discharge vesicular contents for recycling.

MATERIALS AND METHODS

Experimental animals

Yorkshire pigs, raised at the Iowa State University Animal Nutrition Farm, were used for this experiment. Newborn pigs, 1-8 days of age, were killed and decapitated. Pituitary glands were immediately removed and cut in sagittal half. Animal care and experimental protocols were in accordance with the guidelines and approval of the Iowa State University Committee on Animal Care.

Tissue preparation

The pituitary gland was cut into less than 1mm cubes and each half part of the gland was exposed to PBS and L-692,585 for 90 seconds (Fig. 1).

1. TEM methods to optimize structure

The minced sagittal half of the pituitary gland was fixed in 4.0% paraformaldehyde and 2.5% glutaraldehyde for 2 h and then transfer to 1.0% osmium tetroxide for 1 h. During the last step of graded acetone dehydration (25%-100%), tissue was allowed to warm (24˚C) for infiltration and embedding in an Epon-Araldite resin and cut at 40-70 nm.

2. TEM methods for antibody localization

The other minced sagittal half of the pituitary gland was fixed in 2.0% paraformaldehyde and 0.02% glutaraldehyde for 2 h and transfer to 0.15 M glycine for 1 h to bind free aldehyde groups. Then, tissue was dehydrated in graded methanol (25-90%, a drop at a time and lower temperature from 2 ˚C to -20 ˚C), infiltrated and embedded in Unicryl resin. Sections were cut at 40-70 nm.

Immunogold labeling and staining

Grids with sections were incubated on ovalbumin for 10 min, followed by overnight incubation at 2 ˚C with anti-porcine GH antibody raised in rabbit for the primary antibody. After washing in 0.01M PBS, grids were incubated at room temperature on gold conjugated rabbit IgG raised in goat for 1 h. Sections were stained with 2% uranyl acetate for 15 min and then with lead citrate for 40 seconds. The optimum concentration for the antibody was determined by systematically varying the concentration until minimal background was obtained. Controls, substituted PBS for the primary antibody, and labeled with only the

protein A-gold conjugated secondary antibody, showed minimal background labeling. Thin sections were examined at 80 kV in a Jeol JEM-100CXII electron microscope. Images were recorded on Kodak SO-163 electron image film.

Quantitative analysis

Images acquired by scanning were printed on inkjet printer and counted three categories of vesicles (filled, empty and partly empty) independently by two persons. Each category of counted vesicles was represented by the number of vesicle on a μm^2.

Statistical analysis

Mean of the number of vesicle on a μm^2 of each category were obtained for PBS and L-692,585 treated groups. Data are expressed as the mean ± S.E.M. All data were subjected to analysis of variance (ANOVA) to establish whether significant differences ($P < 0.001$ or $P < 0.05$) were present, in which case P values for pair-wise differences between groups were calculated by Student's t test.

RESULTS

Number of secretory vesicles in GH cells after secretion

Control pituitary cells exposed to PBS contained more than twice as many filled vesicles than did the stimulated cells exposed to L-692,585 (4.9 ± 0.21 in control, 2.3 ± 0.23 in stimulated; significantly changed at $P < 0.001$) , whereas simulated cells contained nearly twice as many empty vesicles (0.6 ± 0.13 in control, 1.2 ± 0.16 in stimulated; significantly

changed at $P < 0.05$) and 2.5 × more partly empty vesicles than did control cells (1.1 ± 0.08 in control, 2.6 ± 0.12 in stimulated; significantly changed at $P < 0.001$) (Table 1.). However, there was no significant difference in total number of vesicles between control and stimulated pituitary cells (Fig. 2). A remarkable increase in number of empty and partly empty vesicles was shown in stimulated cells compared to control cells (Fig. 3A, B). There was also a marked change in number of GH vesicles after secretion and notable localization of electron dense particles in GH vesicles in control and stimulated cells. (Fig. 4A, B). Combining with the earlier AFM study in porcine GH cells, this TEM study confirms that membrane boundary secretory vesicles in GH cells transiently dock and fuse at the fusion pore to release vesicular contents by no loss of the number of secretory vesicle.

DISCUSSION

Recently, final step of exocytosis has been reconsidered against the commonly accepted process, "total fusion" involving total incorporation of the secretory vesicle membrane with the cell plasma membrane and the compensatory retrieval of excess membrane by endocytosis at a later time. The majority of the electrophysiological measurements, TEM and AFM studies demonstrate that membrane bounded secretory vesicles transiently dock and fuse at the fusion pore to release their vesicular contents. A step increase in membrane capacitance also may be due to secretory vesicles undergoing "transient fusion" at the plasma membrane during exocytosis. In slow secretory cells like the pancreatic cell in mice, membrane capacitance involves only transient fusion events after stimulation of secretion. In fast secretory cells such as nerve or mast cells, the number of secretory vesicles fusing at the plasma membrane at one time may be greater than in the pancreatic cells. The sequential

fusion of secretory vesicles before their dissociation from the plasma membrane more likely encounters a step increase in plasma membrane capacitance in nerve cells that also require a rapid and selective retrieval of vesicle membrane in addition to rapid fusion of synaptic vesicles at the presynaptic membrane. Considering the importance of both time and energy in the cellular process, exocytosis in neurons or neuroendocrine cells is rather dependent on transient fusion of vesicles at the membrane fusion pore. Continuous exocytosis followed by membrane retrieval would permit endocrine cells to efficiently maintain secretory activity for long period time maintaining the cell membrane area constant. In particularly, rat pituitary GH cells have the ability to undergo continuous exocytosis and membrane retrieval that persist in whole-cell recording (Gordan Kilic et al., 2001, Gloria Majo et al., 1998) suggest similar secretory mechanisms for synaptic vesicles and secretory organelles in both neuronal and endocrine cells which have a highly regulated secretory pathway for intracellular communication by secretory vesicles that fuse with the plasma membrane in response to a physiological stimulus by showing the similarities of synaptic proteins in anterior pituitary with in the nerve terminal. Although several secretory vesicles-associated proteins have been implicated in exocytosis, none have incorporation at the plasma membrane for total fusion. From our study using GH cells, it was determined 90 seconds exposure of 20 μM L-692,585 (GH-secretagogue) for stimulation of GH secretion, which was ideal in inducing rapid and effective exocytosis. Intracellular signal transduction of GH-secretagogue (GHS) is undergoing a phosphoinositol-protein kinase C pathway that induces intracellular Ca^{2+} accumulation and depolarization, leading to exocytosis of GH-containing vesicles. A step increase in plasma membrane capacitance of GH cells, therefore, may be caused by rapid transient fusion based on the AFM observation (Cho et al., 2002) that shows the presence of

"pit" and "depression" at the GH cell plasma membrane and 40% increase in the size in GHS exposed GH cells. No loss of GH secretory vesicle number after exocytosis in our results extended the AFM study consistent with a mechanism that vesicles transiently dock and fuse at the fusion pore to release vesicular contents following stimulation of secretion. Based on current findings, transient fusion of secretory vesicles at the plasma membrane fusion pore may be the dominant rule in exocytosis. It would be of great interests to understand the mechanism of membrane fusion during whole cycle of endocytosis and exocytosis in secretory vesicles.

REFERENCES

Betz WJ and Angleeson JK. 1998 The synaptic vesicle cycle. Annu. Rev. Physiol. 60:347-363.

Ceccarelli B, Hurlbut WP and MauroA. 1973 Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J. Cell Biol. 57:499-524.

Cho S-J, Jeftinija K, Glavaski A, Jeftinija S, Jena BP and Anderson LL. 2002 Structure and dynamics of the fusion pores in live GH-secreting cells revealed using atomic force microscopy. Endocrinology 143:1144-1148.

Cho, S-J, Cho J and Jena BP. 2002 The number of secretory vesicles remains unchanged following exocytosis. Cell Biol. Int.26:29-33.

Cho S-J, Quinn AS, Stromer MH, Dash S, Cho J, Taatjes DJ and Jena BP. 2002 Structure and dynamics of the fusion pore in live cells. Cell Biol. Int. 26:35-42.

Hauser, JE and Reese TS. 1973 Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. 57:315-344.

Kilic G, Angleson JK, Cochilla AJ, Nussinovitch I and Betz WJ. 2001 Sustained stimulation of exocytosis triggers continuous membrane retrieval in rat pituitary somatotrophs. J. Physiol. 532:771-783.

Lawson D, Fewtrell C, Gomperts B, Raff MC.1975 Anti-immunoglobulin-induced histamine secretion by rat peritoneal mast cells studied by immunoferritin electron microscopy. J. Exp. Med. 142:391-401.

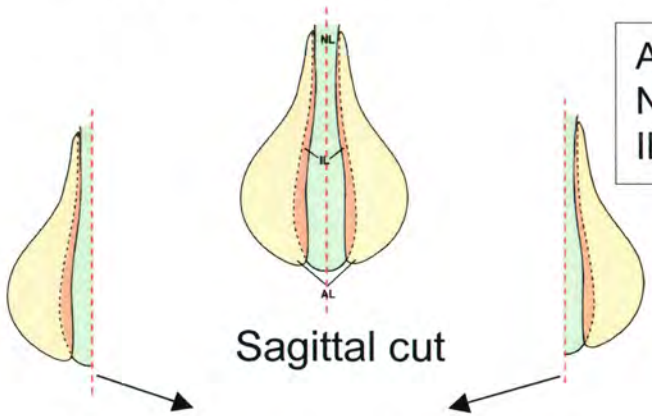
Majo G, Aguado F, Blasi J and Marsal J. 1998 Synaptobrevin isoforms in secretory granules and synaptic-like microvesicles in anterior pituitary cells Life Sci. 62:607-616.

Platter H, Artalejo AR, Neher E. 1997 Ultrastructural organization of bovine chromaffin cell cortex-analysis by cryofixation and morphometry of aspects pertinent to exocytosis. J. Cell Biol. 139:1709-1717.

Schneider SW, Sritharan KC, Geibel JP, Oberleithner H, and Jena BP. 1997 Surface dynamics in living acinar cells imaged by atomic force microscopy: Identification of plasma membrane structures involved in exocytosis Proc. Natl.Acad. Sci. USA 94:316-321.

Figure 1. Description of experimental and control groups for comparing the number of secretory vesicles in GH cells of 1 day-old porcine pituitary.

Experimental Design



AL = Anterior Lobe
NL = Neural Lobe
IL = Intermediate Lobe

Sagittal cut

Cut tissue into < 1 mm cubes

PBS

L-692,585

TEM Methods to Optimize Structure	TEM Methods for Antibody Localization

Immunogold labeling

Staining

Table 1. The number of each category of vesicles in control (PBS) and stimulated (L-692,585) cells

	Total no. of vesicles†		No. of filled vesicles†		No. of empty vesicles†		No. of partly empty vesicles†	
	PBS	**L-585**	**PBS**	**L-585**	**PBS**	**L-585**	**PBS**	**L-585**
Mean	6.61	6.08	4.9	2.3	0.6	1.2	1.1	2.6
SEM	0.14	0.21	0.21	0.23	0.13	0.16	0.08	0.12

† Each category of vesicles is on 1 μm^2 of cell area basis

Figure 2. Total number of secretory vesicles in GH cells remains unchanged after exocytosis. Note the changes in number of filled, empty, and partly empty vesicles between control (PBS) and stimulated (L-692,585) cells.

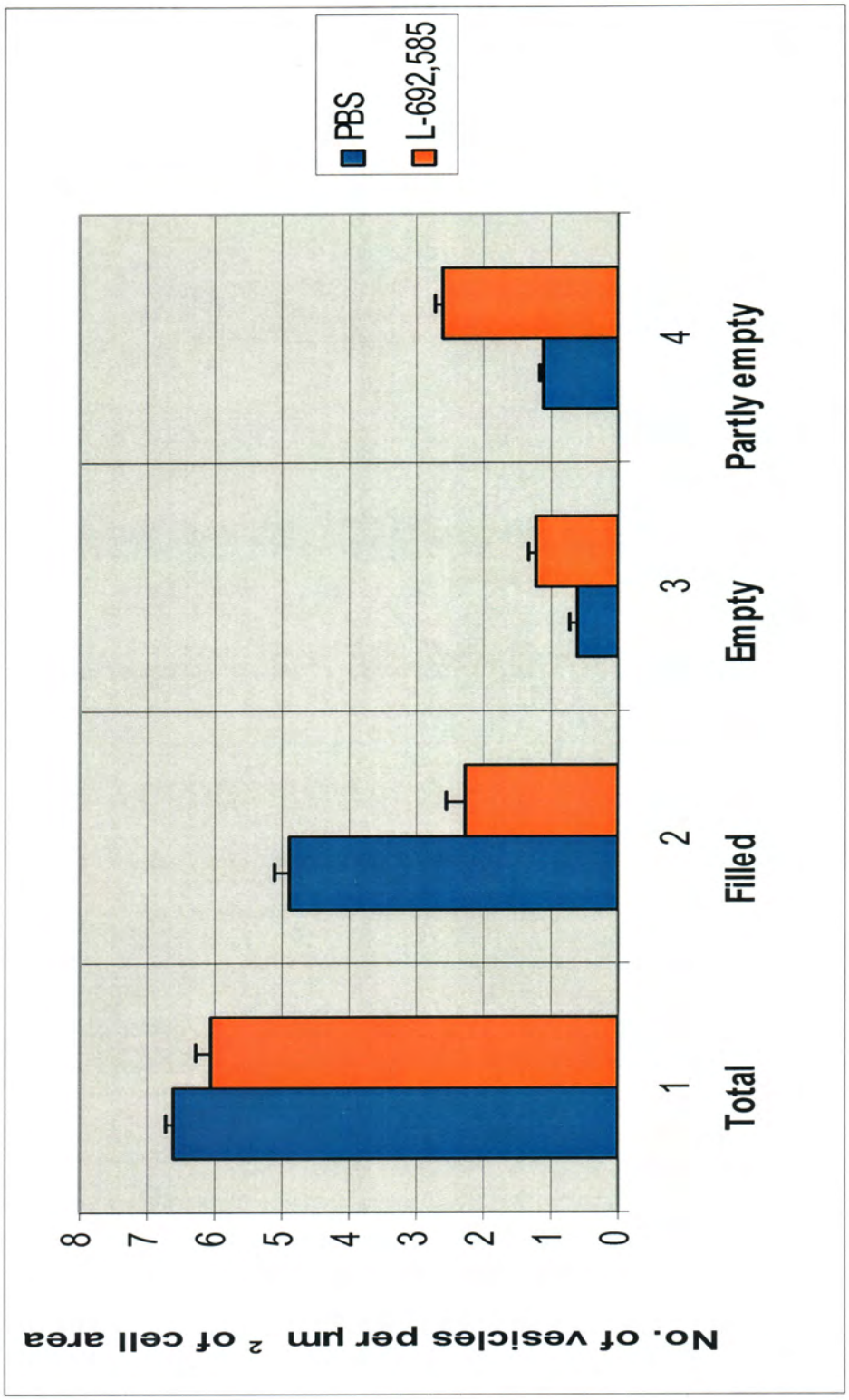
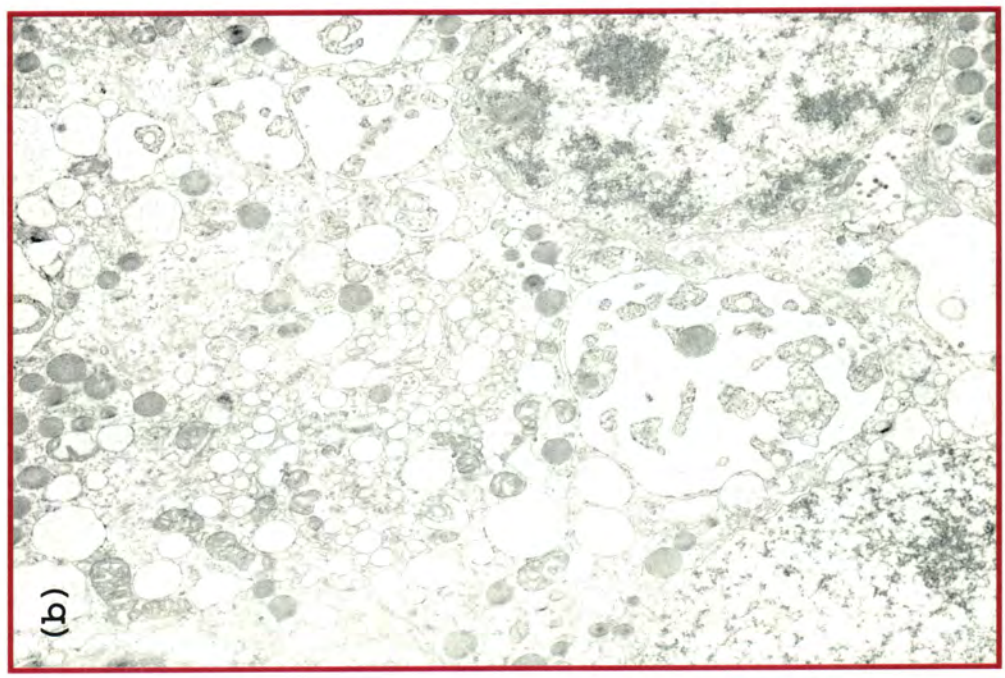

No. of vesicles per µm ² of cell area
0
1
2
3
4
5
6
7
8
1
Total
2
Filled
3
Empty
4
Partly empty
PBS
L-692,585

Figure 3. Images of secretory vesicles in GH cells of porcine pituitary. Note the high number of filled vesicles in control cells exposed to PBS (a), and high number of empty and partly empty vesicles in stimulated cells exposed to L-692,585 (b). Final magnification 13,560 X


(b)

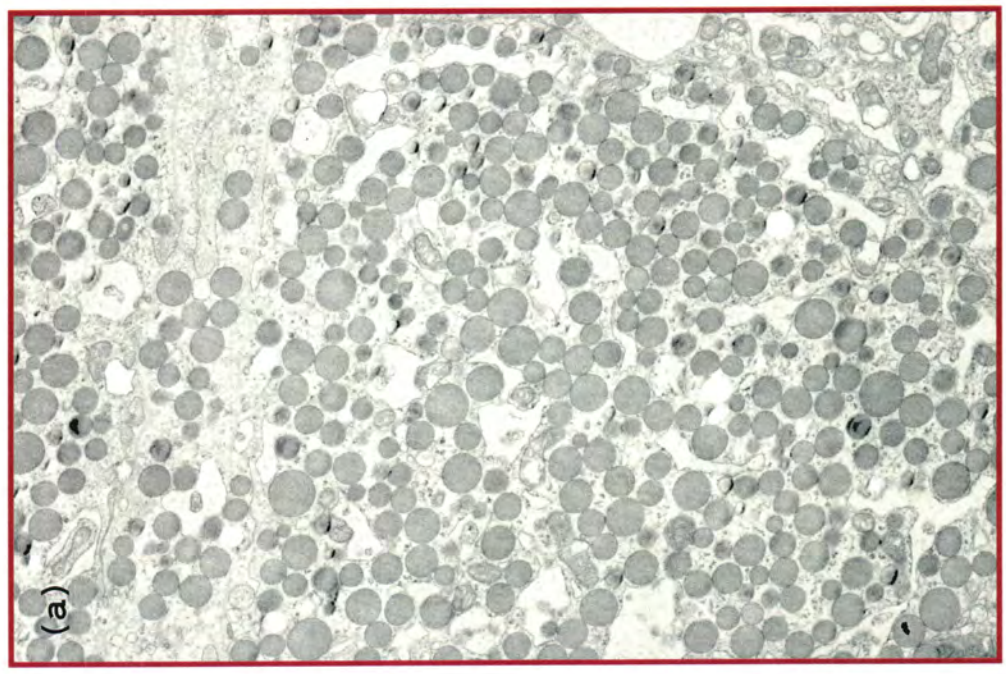
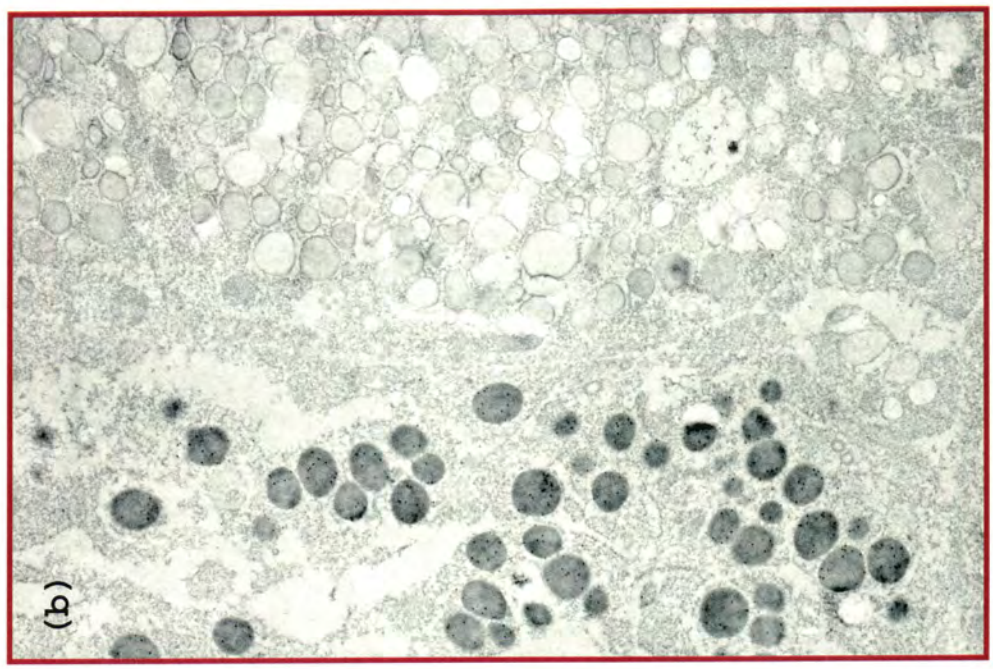

(a)

Figure 4. Immunogold labeled secretory vesicles in GH cells. Note localization of GH is only in electron dense vesicles in both control (a) and stimulated (b) cells. Final magnification 16,541 X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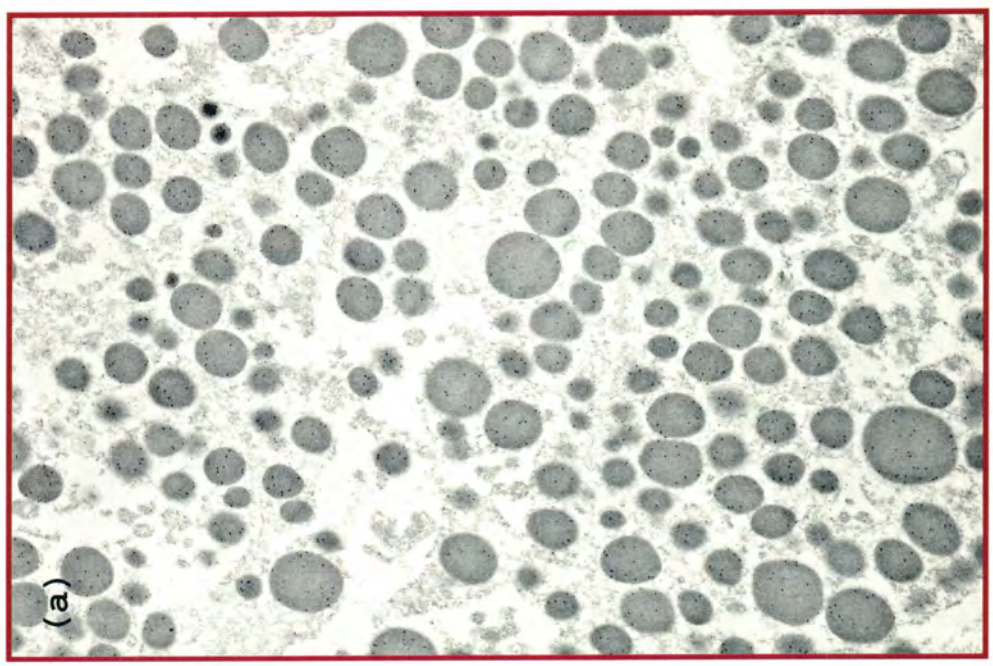
(a)

GENERAL SUMMARY

In the first immunohistochemical study, we identified the spatial distribution patterns of GH secreting cells of the newborn and prepubertal porcine pituitary in light microscopy (LM). Immunoreactive GH cells were round in shape and ranged from 10 to 20 μm in diameter in 1, 42, and 100 day-old pigs. Characteristics of this pattern included a high population of GH cells (43.8 ± 1.2 per 30,495 μm^2, mean ± SEM) in regions 1, 5 (lateral wings of anterior lobe) from proximal (nearest brain or pituitary stalk) to distal (bottom part of gland) and a relatively low population (21.8 ± 1.4 per 30,495 μm^2) of cells in regions 2, 4 (shoulder areas of anterior lobe) from proximal to distal ($P < 0.05$). No significant differences were found among the total GH cell counts per 30,495 μm^2 across the three age groups. However, there was a significant increase of GH cells in region 3 (center of anterior lobe) from proximal to distal (20.1 and 36.4 per 30,495 μm^2, 55.2 %>; $P < 0.05$) in all age groups and a decreasing population of GH cells in region 3 between days 1, 42, and 100 (30.0 and 10.0 per 30,495 μm^2, 33.3 %<; $P < 0.05$). Different immunoreactive density of GH cells across the age may reflect changes in the number of GH vesicles or heterogeneity of GH cells. Our LM observations suggest that there may be regional specificity of cellular differentiation and transformation to facilitate GH secretion in the need for endocrine regulation as the animal ages.

In the second study, transmission electron microscopy (TEM) was used to determine the total number of all categories of secretory vesicles in resting and in GH-stimulated porcine pituitary cells to demonstrate the fate of secretory vesicles after secretion. We identified three categories of vesicles; filled, empty, and partly empty and counted each category of

vesicle is on a μm^2 of cell area basis. Control pituitary cells contained more than twice as many filled vesicles than did the stimulated cells. However, stimulated cells contained nearly twice as many empty vesicles and 2.5 × more partly empty vesicles than did control cells. There was no significant difference in total number of vesicles between control and stimulated pituitary cells. Immunogold labeled secretory vesicles in GH cells revealed that localization of GH is only in electron dense vesicles in control and stimulated cells. Our TEM study reveals no loss of the number of secretory vesicle following exocytosis in GH cells, demonstrating that membrane-bound secretory vesicles transiently dock and fuse to discharge vesicular contents for recycling.

APPENDIX A.

TABULAR DATA OF IMMUNOREACTIVE GH CELL-COUNTING IN LIGHT MICROSCOPY

Table 1-4-a. The number of immunoreactive GH cells at proximal (depth) in 1-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	pig1	pig2	pig3	
1	a	40	54	56	50.0
1	b			48	48.0
1	c	40	45	50	45.0
2	a	0	35	31	22.0
2	b		15	20	17.5
2	c	1	2	18	7.0
3	a	10	57	61	42.7
3	b		37	52	44.5
3	c	1	2	32	11.7
4	a	4	33	36	24.3
4	b		12	19	15.5
4	c	0	3	24	9.0
5	a	50	59	48	52.3
5	b			41	41.0
5	c	41	49	43	44.3

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 4-a, page 40.

Table 1-4-b. The number of immunoreactive GH cells at middle (depth) in 1-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	pig1	pig2	pig3	
1	a	39	54	40	44.3
1	b		42	41	41.5
1	c	31	42	32	35.0
2	a	10	47	16	24.3
2	b		21	23	22.0
2	c	1	7	19	9.0
3	a	12	55	50	39.0
3	b		34	39	36.5
3	c	0	8	22	10.0
4	a	17	48	31	32.0
4	b		19	17	18.0
4	c	2	10	9	7.0
5	a	50	54	41	48.3
5	b		41	35	38.0
5	c	42	49	33	41.3

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 4-b, page 40.

Table 1-4-c. The number of immunoreactive GH cells at distal (depth) in 1-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	pig1	pig2	pig3	
1	a	29	32	44	35.0
1	b			40	40.0
1	c	31	40	48	39.7
2	a	15	32	11	21.5
2	b		21	14	17.5
2	c	19	37	15	23.7
3	a	45	11	47	34.3
3	b		49	49	49.0
3	c	39	57	48	48.0
4	a	20	18	28	22.0
4	b		25	16	20.5
4	c	15	35	23	24.3
5	a	43	39	40	40.7
5	b			36	36.0
5	c	31	37	38	35.3

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 4-c, page 40.

Table 1-5-a. The number of immunoreactive GH cells at proximal (depth) in 42-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	Pig4	Pig5	Pig6	
1	a	56	55	46	52.3
1	b	46			46.0
1	c	11	49	41	33.7
2	a	54	25	31	36.7
2	b	19	9	30	19.3
2	c	2	3	2	2.3
3	a	59	41	26	42.0
3	b	9	5	3	5.7
3	c	4	3	1	2.7
4	a	44	29	25	32.7
4	b	13	15	11	13.0
4	c	8	2	1	3.7
5	a	56	42	43	47.0
5	b	39			39.0
5	c	26	36	37	33.0

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 5-a, page 42.

Table 1-5-b. The number of immunoreactive GH cells at middle (depth) in 42-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	Pig4	Pig5	Pig6	
1	a	55	57	42	51.3
1	b	53	51	39	47.7
1	c	45	51	25	40.3
2	a	45	40	31	38.7
2	b	26	17	19	20.7
2	c	42	7	2	17.0
3	a	47	51	25	41.0
3	b	25	23	20	22.7
3	c	54	13	2	23.0
4	a	41	34	30	35.0
4	b	21	19	15	18.3
4	c	49	9	3	20.3
5	a	51	56	41	49.3
5	b	43	53	37	44.3
5	c	41	49	28	39.3

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 5-b, page 42.

Table 1-5-c. The number of immunoreactive GH cells at distal (depth) in 42-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	Pig4	Pig5	Pig6	
1	a	49	51	54	51.3
1	b	36	53	50	46.3
1	c	33	49	49	43.7
2	a	48	39	42	43.0
2	b	21	29	25	25.0
2	c	31	10	19	20.0
3	a	15	49	24	29.3
3	b	45	53	29	42.3
3	c	48	39	15	34.0
4	a	41	40	39	40.0
4	b	18	30	26	24.7
4	c	29	9	15	17.7
5	a	41	55	49	48.3
5	b	39	54	45	46.0
5	c	31	50	40	40.3

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 5-c, page 42.

Table 1-6-a. The number of immunoreactive GH cells at proximal (depth) in 100-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	Pig7	Pig8	Pig9	
1	a	31	50	52	44.3
1	b	17	46	55	39.3
1	c	8	45	45	32.7
2	a	28	18	15	20.3
2	b	23	16	11	16.7
2	c	0	5	0	1.7
3	a	49	15	0	21.3
3	b	17	4	0	7.0
3	c	4	1	1	2.0
4	a	24	20	15	19.7
4	b	33	19	13	21.7
4	c	9	8	0	5.7
5	a	34	53	47	44.7
5	b	44	47	51	47.3
5	c	40	45	42	42.3

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 6-a, page 44.

Table 1-6-b. The number of immunoreactive GH cells at middle (depth) in 100-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	Pig7	Pig8	Pig9	
1	a	67	31	63	53.7
1	b	64	28	63	51.7
1	c	59	47	52	52.7
2	a	54	22	39	38.3
2	b	48	2	32	27.3
2	c	21	0	10	10.3
3	a	39	15	10	21.3
3	b	15	13	3	10.3
3	c	42	22	0	21.3
4	a	42	27	32	33.7
4	b	44	16	32	30.7
4	c	21	11	15	15.7
5	a	47	36	62	48.3
5	b	46	39	57	47.3
5	c	48	32	50	43.3

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 6-b, page 44.

Table 1-6-c. The number of immunoreactive GH cells at distal (depth) in 100-day old pig pituitary section

		No. of immunoreactive GH cells per 30,495 μm^2			mean
Region	Position	Pig7	Pig8	Pig9	
1	a	38	50	63	50.3
1	b	24	48	67	48.3
1	c	21	37	55	42.0
2	a	41	18	46	36.3
2	b	57	19	31	35.3
2	c	35	7	25	29.0
3	a	51	16	21	25.8
3	b	39	28	14	28.2
3	c	39	34	26	30.0
4	a	42	18	33	32.0
4	b	62	26	34	35.8
4	c	60	15	29	37.7
5	a	28	39	55	37.7
5	b	23	45	48	39.7
5	c	43	48	45	42.0

Note that blank cells present no value available due to the small size of section.
Refer to Chapter 1, Figure 6-c, page 44.

APPENDIX B.

TABULAR DATA OF SECRETORY VESICLE COUNTING OF GH CELLS IN TRANSMISSION ELECTRON MICROSCOPY

Table 1-a. Total number of secretory vesicles in control GH cells

No. of negative images	Total corrected area (μm^2)	No. of secretory vesicles per μm^2		mean
		Count 1	Count 2	
c31393	43.4	8.5	8.1	8.3
c31394	70.8	8.4	6.4	7.4
c31396	124	6.3	5.7	6.0
c31397	59.4	6.9	6.5	6.7
c31398	71.2	6.4	6.0	6.2
c31453(less dense)	47.9	7.0	6.3	6.7
c31453(more dense)	16.8	6.9	5.9	6.4
c31454	76	6.0	6.2	6.1
c31455	69.5	7.5	6.9	7.2
c31456	71.8	6.8	6.3	6.6
c31457	57.4	6.6	6.4	6.5
c31458	64.4	7.0	6.7	6.9
c31459	45.9	7.6	6.9	7.3
c31460	57.2	4.9	4.7	4.8
c31461	67.8	6.2	5.9	6.1
c31462	79.8	7.0	6.8	6.9
mean		6.9	6.3	6.6
SEM		0.16	0.12	0.14

Refer to Chapter 2, Table 1, Page 60.

Table 1-b. Total number of secretory vesicles in GH cells exposed to L-692,585

No. of negative image	Total corrected area (μm^2)	No. of secretory vesicles per μm^2		mean
		Count 1	Count 2	
s31403	60.3	4.9	5.9	5.4
s31463	51.5	6.8	5.6	6.2
s31464	63.2	5.4	4.9	5.2
s31465	53.3	4.5	3.9	4.2
s31466	40	7.1	7.4	7.2
s31467	59.5	6.2	5.4	5.8
s31468	66.9	6.0	5.8	5.9
s31469	85.2	6.8	6.7	6.8
s31470	72.5	6.5	5.8	6.1
s31471	49.3	6.3	5.6	6.0
s31472	71.9	8.0	7.6	7.8
mean		6.21	5.87	6.04
SEM		0.21	0.21	0.21

Refer to Chapter 2, Table 1, Page 60.

Table 2-a Number of filled vesicles in control GH cells

No. of negative image	No. of filled vesicles		No. of vesicles per μm^2		mean
	Count 1	Count 2	Count 1	Count 2	
c31393	229	219	5.3	5.1	5.2
c31394	369	348	5.2	4.9	5.1
c31396	474	428	3.8	3.5	3.6
c31397	211	200	3.6	3.4	3.5
c31398	293	268	4.1	3.8	3.9
c31453(less dense)	282	245	5.9	5.1	5.5
c31453(more dense)	87	72	5.2	4.3	4.7
c31454	352	337	4.6	4.4	4.5
c31455	370	361	5.3	5.2	5.3
c31456	372	354	5.2	4.9	5.1
c31457	308	302	5.4	5.3	5.3
c31458	401	382	6.2	5.9	6.1
c31459	257	248	5.6	5.4	5.5
c31460	227	217	4.0	3.8	3.9
c31461	345	326	5.1	4.8	5.0
c31462	506	479	6.3	6.0	6.2
mean	317.7	299.1	5.0	4.7	4.9
SEM	20.23	19.51	0.22	0.20	0.21

Refer to Chapter 2, Table 1, Page 60.

Table 2-b Number of filled vesicles in GH cells exposed to L-692,585

No. of negative image	No. of filled vesicles		No. of filled vesicles per μm^2		mean
	Count 1	Count 2	Count 1	Count 2	
s31403	195	185	3.2	3.1	3.2
s31463	49	46	1.0	0.9	0.9
s31464	72	70	1.1	1.1	1.1
s31465	72	72	1.4	1.4	1.4
s31466	117	120	2.9	3.0	3.0
s31467	130	123	2.2	2.1	2.1
s31468	113	107	1.7	1.6	1.7
s31469	254	227	3.0	2.7	2.8
s31470	184	171	2.5	2.4	2.5
s31471	112	103	2.3	2.1	2.2
s31472	298	281	4.1	3.9	4.0
mean	145.1	136.8	2.3	2.2	2.3
SEM	19.2	17.35	0.23	0.22	0.23

Refer to Chapter 2, Table 1, Page 60.

Table 3-a Number of empty vesicles in control GH cells

No. of negative image	No. of empty vesicles		No. of empty vesicles per μm2		mean
	Count 1	Count 2	Count 1	Count 2	
c31393	73	71	1.68	1.64	1.66
c31394	148	28	2.09	0.4	1.25
c31396	65	67	0.52	0.54	0.53
c31397	110	99	0.85	1.67	1.26
c31398	56	55	0.79	0.77	0.78
c31453(less dense)	11	12	0.12	0.25	0.19
c31453(more dense)	6	5	0.65	0.3	0.48
c31454	62	54	0.82	0.71	0.77
c31455	66	51	0.95	0.73	0.84
c31456	28	23	0.39	0.35	0.37
c31457	19	16	0.33	0.28	0.31
c31458	16	14	0.25	0.22	0.24
c31459	29	28	0.63	0.61	0.62
c31460	23	25	0.4	0.44	0.42
c31461	23	22	0.34	0.32	0.33
c31462	12	13	0.15	0.16	0.16
mean	46.7	36.4	0.7	0.6	0.6
SEM	7.91	5.57	0.12	0.11	0.13

Refer to Chapter 2, Table 1, Page 60.

Table 3-b Number of empty vesicles in GH cells exposed to L-692,585

No. of negative image	No. of empty vesicles		No. of empty vesicles per μm^2		mean
	Count 1	Count 2	Count 1	Count 2	
s31403	32.0	29.0	0.5	0.5	0.5
s31463	131.0	121.0	2.5	2.4	2.5
s31464	108.0	95.0	1.7	1.5	1.6
s31465	34.0	19.0	0.6	0.4	0.5
s31466	100.0	87.0	2.5	2.2	2.3
s31467	33.0	23.0	0.6	0.4	0.5
s31468	84.0	74.0	1.3	1.1	1.2
s31469	104.0	94.0	1.2	1.1	1.2
s31470	101.0	90.0	1.4	1.2	1.3
s31471	24.0	23.0	0.5	0.5	0.5
s31472	101.0	92.0	1.4	1.3	1.3
mean	77.5	67.9	1.3	1.1	1.2
SEM	10.2	9.7	0.2	0.2	0.16

Refer to Chapter 2, Table 1, Page 60.

Table 4-a Number of partly empty vesicles in control GH cells

No. of negative image	No. of partly empty vesicles		No. of partly empty vesicles per μm^2		mean
	Count 1	Count 2	Count 1	Count 2	
c31393	69	60	1.59	1.38	1.49
c31394	77	78	1.08	1.1	1.09
c31396	237	214	1.91	1.73	1.82
c31397	89	85	1.5	1.43	1.47
c31398	107	101	1.5	1.42	1.46
c31453(less dense)	46	45	0.96	0.94	0.95
c31453(more dense)	18	22	1.07	1.31	1.19
c31454	42	79	0.55	1.04	0.8
c31455	86	67	1.24	0.96	1.1
c31456	91	73	1.27	1.02	1.15
c31457	50	48	0.87	0.84	0.86
c31458	35	33	0.54	0.51	0.53
c31459	64	40	1.39	0.87	1.13
c31460	29	24	0.51	0.42	0.47
c31461	55	51	0.81	0.75	0.78
c31462	40	47	0.5	0.59	0.55
mean	70.9	66.7	1.11	1.0	1.1
SEM	8.17	7.19	0.09	0.07	0.08

Refer to Chapter 2, Table 1, Page 60.

Table 4-b Number of partly empty vesicles in GH cells exposed to L-692,585

No. of negative image	No. of partly empty vesicles		No. of partly empty vesicles per μm^2		mean
	Count 1	Count 2	Count 1	Count 2	
s31403	67.0	140.0	1.11	2.32	1.72
s31463	168.0	121.0	3.26	2.35	2.81
s31464	162.0	146.0	2.56	2.31	2.44
s31465	134.0	116.0	2.51	2.18	2.35
s31466	65.0	89.0	1.62	2.22	1.92
s31467	208.0	175.0	3.5	2.94	3.22
s31468	202.0	206.0	3.02	3.08	3.05
s31469	225.0	248.0	2.64	2.91	2.78
s31470	183.0	160.0	2.52	2.21	2.37
s31471	175.0	152.0	3.55	3.08	3.32
s31472	173.0	176.0	2.41	2.45	2.43
mean	160.2	157.2	2.6	2.6	2.6
SEM	11.7	9.8	0.2	0.1	0.12

Refer to Chapter 2, Table 1, Page 60.

REFERENCES

Abbott CR, Rossi M, Wren AM, Murphy KG, Kennedy AR, Stanley SA, et al. 2001 Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. Endocrinology 142:3457-3463.

Adams EF, Huang B, Buchfelder M. 1998 presence of growth hormone secretagogue receptor messenger ribonucleic acid in human pituitary tumors and rat GH3 cells. J. Clin. Endocrinol. Metab. 83:638-642.

Alvarez DE, Toledo G, Fernandez-Chacón R, Fernandez JM. 1933 Release of secretory products during transient vesicle fusion. Nature 363:554-558.

Avart E, Maccario M, Di Vito L, Groglio F, Benso A, Gottero C, Camanni F, Ghigo E. 2002 Endocrine activity of ghrelin, a natural growth hormone secretagogue (GHS), in human: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. J. Clin. Endocrinol. Metab. 86:1169-1174.

Baker BL, 1974 Functional cytology of the hypophysial pars distalis and pars intermedial. Handbook Physiol. Sect. Endocrinol. 4:45-80.

Baumbach WR, Horner DL, Logan JS. 1989 The growth hormone-binding protein in rat serum is an alternatively spliced form of the rat growth hormone receptor. Genes Dev. 3:1199-1205.

Bendnarek MA, Feighner VA, Howard AD, Van Der Ploeg LH, Heck JV. 2000 Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor. J. Med. Chem. 43:4370-4376.

Bennett PA, Levy A, Sophokleous S, Robinson ICAF, Lightman SL. 1995 Hypothalamic growth hormone receptor gene expression in the rat. J. Endocrinol. 147:225-234.

Bowers CY, Momany F, Reynolds GA, Hong A. 1984 On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. Endocrinology 114:1537-1545.

Bowers CY, Reynolds GA, Durham D, Marrera CM, Pezzoli SS, Thorner MO. 1990 Growth hormone (GH)-releasing peptide stimulates GH release in normal men and acts synergistically with GH-releasing hormone. J. Clin. Endocrinol. Metabol. 70:975-982.

Bowers CY, Chang JK, Fong TTW. 1977 A synthetic pentapeptide which specifically release GH, in vitro. In: Program & Abstracts of the 59th meeting of the Endocrine Society p232

Burton KA, Kabigting EB, Clifton DK, Steiner RA. 1992 Growth hormone receptor messenger ribonucleic acid distribution in the adult male rat brain and its co-localization in hypothalamic somatostatin neurons. Endocrinology 131:958-963.

Chan YY, Steiner RA, Clifton DK. 1996 Regulation of hypothalamic neuropeptide-Y neurons by growth hormone in the rat. Endocrinology 137:1319-1325.

Chan YY, Steiner RA, Clifton DK. 1996 Regulation of hypothalamic neuropeptide-Y neurons by growth hormone in the rat. Endocrinology 137:1319-1325.

Cheng K, Chan W-S, Butler B, Barreto A, Smith RG. 1991 Evidence for a role of protein kinase-C in His-D Trp-Ala-Trp-D-Phe-Lys-NH_2-induced growth hormone release from rat primary pituitary cells. Endocrinology 129:3337-3342.

Chihara K, Minamitani N, Kaji H, Arimura A, Fujita T. 1981 Intravetricularly injected growth hormone stimulates somatostatin release into rat hypophysial portal blood. Endocrinology 109:2279-2281.

Cho S-J, Jeftinija K, Glavaski A, Jeftinija S, Jena BP, and Anderosn LL. 2002a Structure and dynamics of the fusion pores in live GH-secreting cells revealed using atomic force microscopy. Endocrinology 143:1144-1148.

Cho SJ, Quinn AS, Stromer MH, Dash S, Cho J, Taatjes DL, Jena BP. 2002b Structure and dynamics of the fusion pore in live cells. Cell Biol. Int. 26:35-42.

Cho SJ, Wakade A, Pappas GD, Jena BP. New structure involved in transient membrane fusion and exocytosis. Ann NY Acad. Sci.

Chomczynski P, Downs TR, Frohman LA. 1988 Feedback regulation of growth hormone releasing hormone gene expression by GH in rat hypothalamus. Mol. Endocrinol. 2:236-241.

Clark RG, Carlsson LMS, Robinson ICAF. 1988 Growth hormone (GH) secretion in the conscious rat: negative feedback of GH on its own release. J. Endocrinol. 119:201-209.

Clark RG, Carlsson LMS, Trojnar J, Robinson ICAF. 1989 The effects of a growth hormone-releasing peptide and growth hormone-releasing factor in conscious and anesthetized rats. J. Neuroendocrinol. 1:249-255.

Conklin JL, 1962 The development of the human fetal adenohypophysis. Anat. Rec. 106:79-92.

Conway S, McCann SM, Krulich L. 1985 On the mechanism of growth hormone autofeedback regulation: possible role of somatostatin and growth hormone release factor. Endocrinology 117:2284-2292.

Conway S, McCann SM, Krulich L. 1985 On the mechanism of growth hormone autofeedback regulation: possible role of somatostatin and growth hormone release factor. Endocrinology 117:2284-2292.

Dacheux F. 1984 Functional differentiation of the anterior pituitary cells in the fetal pig. Cell Tissue Res. 235:623-633.

Data Y, Kojima M, Hosoda H, Sawaguchi A, Mondal M. 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141:4255-4261.

Denf C, Baes M, Schramme C, Swennen L. 1984 The role of cell-cell communication in neuropeptide-stimulated and dopamine-inhibited porlactin release. In "Hormonal Control of the Hypothalamo-Pituitary-Gonaadal Axis" Plenum, New York. pp355-366.

Dickson SL, Leng G, Robinson ICAF. 1993 Systemic administration of growth hormone-releasing peptide (GHRP-6) activates hypothalamic arcuate neurons. Neuroscience 53:303-306.

Dickson SL, Leng G, Dyball REJ, Smith RG. 1995 Central action of peptide and non-peptide growth hormone secretagogues in the rat. Neuroendocrinology 61:36-43.

Eva C, Luisa MS, Rosa S, Robert VC, Felipe FC, Carlos D. 1998 Interaction between leptin and neuropeptide Y on in vivo growth hormone secretion. Neuroendocrinology 68:187-191.

Frick GP, Leonard JL, Goodman HM. 1990 Effect of hypophysectomy on growth hormone receptor gene expression in rat tissue. Endocrinology 126:3076-3082.

Fujii Y, Conoi T, Yamada Y, Chihara K, Inagaki N, Seino S. 1994 Somatostatin receptor subtype SSTR2 mediates the inhibition of high voltage activated calcium channels by somatostatin and its analogue SMS201-995. FEBS Lett. 355:117-120.

Bielańska-Osuchowaska Z, and Liwska J. 1975 Studies on development of the adenhpophysis in the domestic pig Folia Morphol. 34:143-149.

Kandel ER, Schwartz JH, Jessell TM. 1995 Essentials of neural science and behavior Appleton & Lange, Norwalk, Connecticut 282-286

Korbonits M, Kojima M, Kangawa K, Grossman AB. 2001 Presence of ghrelin in normal and adenomatous human pituitary. Endocrine 14:101-104.

Gurd W, Parent G, Eniojukan R, Bowers CY, Tannenbaum GS. 2001 Interrelationship between the novel peptide ghrelin and somatostatin/GHRH in regulation of pulsatile GH secretion. Proceedings of the 83rd Annual Meeting of The Endocrine Society. Denver, CO. Bethesda, MD: The Endocrine Society p72.

Harel Z, Tannenbaum GS. 1992 Synergistic interaction between insulin-like growth factor-I and II in central regulation of pulsatile growth hormone secretion. Endocrinology 131:758-764.

Harvey S, Hull KL, Fraser RA. 1993 Mini-review: Growth hormone: neurocrine and neuroendocrine perspectives. Growth Reg. 3:161-171.

Herlant M, 1964 The cells of the adenohypophysis and their functional significance. Int. Rev. Cytol. 17:299-382.

Hickey GJ, Drisko J, Faidley T., Chang C, Anderson LL, Nicolich S, McGuire L, Rickes E, Krupa D, Feeney W, Friscino B, Cunningham P, Fraizer E, Chen H, Laroque P, Smith RG 1996 Mediation by the central nervous system is critical to the *in vivo* activity of the GH secretagogue L-692,585. J. Endocrinol. 148:371-380.

Ilona AB, Clement CC, David SW, Hongping REN Emilia BK, Joseph LK, Donald KC, Robert AS. 1996 Leptin is a metabolic signal to the reproductive system. Endocrinology 137:3144-3147.

Jamieson JD. 1972 Transport and discharge of exportable proteins in pancreatic exocrine cells: in vitro studies. In: Bronner F, Kleinzeller a eds. Current Topics in Membranes and Transport. Academic Press. New York and London. pp273-338.

Kalra SP, Sahu A, Kalra PS, Crowley WR. 1990 Hypothalamic neuropeptide Y: A circuit in the regulation of gonadotropin secretion and feeding behavior. Ann. N.Y. Acad. Sci. 611:273-283.

Korbonits M, Kojima M, Kangawa K, Grossman AB. 2001 Presence of ghrelin in normal and adenomatous human pituitary. Endocrine 14:101-104.

Korbonits M, Bustin SA, Kojima M, Jordan S, Adams EF, Lowe DG, Kangawa K, Grossman AB. 2001 The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. J. Clin. Endocrinol. Metab. 86:881-887.

Lanzi R, Tannenbaum GS. 1992 Time course and mechanism of growth hormone negative feedback effect on its own spontaneous release. Endocrinology 130:780-788.

Li CH. 1972 Hormones of the adenohypophysis. Proc. Am. Physiol. Soc. 116:365-382.

Linial M, Parnas D. 1996 Deciphering neuronal secretion: tools of the trade. Biochem. Biophys.Acta. 1286:117-152.

Lobie PE, Garcia-Aragon J, Lincoln DT, Barnard R, Wilcox JN, Waters MJ.

1993 Localization and ontogeny of growth hormone receptor gene expression in the central nervous system. Dev. Brain Res. 74:225-233.

Malozowski S, Hao EH, Ren SG, Marin G, Liu L, Southers JL, Merriam GR. 1991 Growth hormone (GH) responses to hexapeptide GH-releasing peptide and GH-releasing hormone (GHRH) in the cytomolgus macaque: evidence for non-GHRH-mediated responses. J. Clin.Endocrinol. Metab. 73:314-317.

Mayo KE, Godfrey PA, Suhr ST, Kulik DJ, Rahal JO. 1995 Growth hormone-releasing hormone: synthesis and signaling. Recent Prog. Horm. Res. 50:35-73.

Matsumoto M, Kitajima Y, Iwanami T, Hayashi Y, Tanaka S, Minamitake Y, HosodaH, Kojima M, Matsuo H, Kangawa K. 2001 Structural similarity of ghrelin derivatives to peptidyl growth hormone secretagogues. Biochem. Biophys. Res. Commun. 284:655-659.

Minami S, Kamegai J, Hasgawa O, Sugihara H, Okada K, Wakabayashi I. 1993 Expression of growth hormone receptor gene in rat hypothalamus. J. Neuroendocrinol. 5:691-696.

Monck JR, Oberhause AF, Fernandez JM. 1995 The exocytotic fusion pore interface: a model of the site of neurotransmitter release. Mol. Memb. Biol. 12:151-156.

Mori K, Yoshimoto A, Takaya K, Hosoda H, Ariyasu H, Yahata KMukoyama M, Sugawara A, Hosoda H, Kojima, Kangawa K, Nakao K. 2000 Kidney produces a novel acylated peptide, ghrelin. FEBS Lett. 486:213-216.

Mucciloi G, Papotti M, Locatelli V, Ghigo E, Dghenghi R. 2001 Binding of ^{125}I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J. Endocrinol. Invest. 24:RC7-9.

Nameskéri A, Sétáló G, and Halász B. 1988 Ontogenesis of the three parts of the fetal rat adenohypophysis: A detailed immunohistochemical analysis. Neuroendocrinology 48:534-543.

Nelson WO. 1933 Studies on the anterior hypophysis. I. The development of the hypophysis in the pig (*Sus scrofa*). II. The cytological differentiation in the anterior hypophysis of the foetal pig. Am. J. Anat. 52:307-332.

Noblet J, Herpin P, Dubois S. 1992 Effect of recombination porcine somatotrophin on energy and protein utilization by growing pigs: Interaction with capacity for lean tissue growth. J. Anim. Sci. 70:2471-2484.

Ojeda SR and Griffin JE. 1996 Organization of the endocrine system. In: J.E. Griffin and S.R. Ojeda (Ed). Textbook of Endocrine Physiology.OxfordUniversity Press, New York. Pp3-17.

Pantic V. 1974 The cytology of thyroid cells. Int. Rev. Cytol. 36:153-243.

Patten BM. 1948 Embryology of the Pig. McGraw-Hill Book, New York.

Pellegrini E, Bluet-Pjot MT, Mounier F, Bennett P, Kordon C, Epelbaum J. 1996 Central administration of a growth hormone (GH) receptor mRNA antisense increases

GH pulsatility and decreases hypothalamic somatostatin expression in rats. J. Neurosci. 16:8140-8148.

Prekereis R and Terrian DM. 1997 Brain myosin V is a synaptic vesicle-associated motor protein: evidence for a Ca^{2+}-dependent interaction with the synaptobrevin-synaptophysin complex. J. Cell Biol. 137:1589-1601.

Reisine T, Bell GI. 1995 Molecular biology somatostatin receptors. Endocr. Rev. 16:427-442.

Richman RA, Weiss JP, Hochberg Z, Florini JR. 1981 Regulation of growth hormone release: evidence against negative feedback in rat pituitary cells. Endocrinology 108:2287-2292.

Robinson ICAF, Carmignac DF, Fairhall KM. 1993 Growth hormone (GH) receptors, GH binding protein and GH: an autoregulatory system? Acta Paediatr. Suppl. 391:22-28.

Rogers KV, Vician L, Steiner A, Clifton DK. 1988 The effect of hypophysectomy and growth hormone administration on pre-pro somatostatin messenger ribonucleic acid in the periventricular nucleus of the rat hypothalamus. Endocrinology 122:586-591.

Romeis B 1940 Handbunch der mikroskopischen Anatomie des Menschen Springer, Berlin. Vol 6:3

Rothman JE 1994 Intracellular membrane fusion. Advances in Second Messenger Phosphoprotein. Res. 29:81-96.

Sato M, Frohman LA. 1993 Differential effects of central and peripheral administration of growth hormone (GH) and insulin-like growth factor on hypothalamic GH-

releasing hormone and somatostatin gene expression in GH-deficient dwarf rats. Endocrinology 133:793-799.

Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, et al. 2001 Ghrelin, an endogeneous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50:227-232.

Schartz J. 2000 Intercellular communication in the anterior pituitary. Endocr. Rev. 21:488-513.

Schneider SW, Sritharan KC, Geibel JP, Oberleithner H, Jena BP. 1997 Surface dynamics in living acinar cells imaged by atomic force microscopy: identification of plasma membrane structures involved in exocytosis. Pro. Natl. Acad. Sci. USA 94:316-321.

Sétáló G and Nakane PK. 1972 Studies on the functional differentiation of the cells in fetal anterior pituitary glands of rats with peroxidase-labled antibody method. Anat. Rec. 172:403-404.

Sétáló G and Nakane PK. 1976 Functional differentiation of the fetal anterior pituitary cells in the rat. Endocrinol. Exp. 10:155-166.

Smith WC, KuniyoshiJ, Talamantes F. 1989 Mouse serum growth hormone (GH) binding protein has GH receptor extracellular and substituted transmembrane domains. Mol Endocrinol. 3:984-990.

Smith RG, Van der Ploeg LHT, Cheng K, Hickey GJ, Wyvratt J, MJ, Fisher MH.. 1997 Peptidomimetic regulation of growth hormone (GH) secretion. Endocr. Rev. 13:621-645.

Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE. 1993 SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318-324.

Tannenbaum GS. 1980 Evidence of autoregulation of growth hormone secretion via the central nervous system. Endocrinology 107:2117-2120.

Tannenbaum GS, Bowers CY. 2001 Interactions of growth hormone secretagogues and growth hormone-releasing hormone/somatostatin. Endocrine 13:21-27.

Volante M, Allia E, Gualiotta P, Funaro A, Broglio F, Deghenghi R, Mucciolo G, Ghigo E, Papotti M. 2002 Expression of ghrelin and of GHS receptor by pancreatic islet cells and related endocrine tumors. J. Clin. Endocrinol. Metab. 87:1300-1308.

Wingstrand KG. 1951 Structure and Development of Avian Piuitary."CW Gleerup, Lund.

Willoughby JO, Menadue M, Zeegers P, Wise PH, Oliver JR. 1980 Effects of human growth hormone secretion of rat growth hormone. J. Endocrinol. 86:165-169.

Wood SC, Seeley RJ, Porte Jr D, Schwarz MW. 1998 Signals that regulate food intake and energy homeostasis. Science 280:1378-1383.

Zhai Q, Lai Z, Roos P, Nuberg F. 1994 Characterization of growth hormone binding sites in rat brain. Acta Paediatr. Suppl. 406:92-95.

Zhang Y, Proenca R, Maffei M, Marone M, Leopold L, Friedman JM. 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425-423.

Zheng H, Bailey ART, Jiang M-H, Honda K, Chen HY, Trumbauer ME, et al. 1997 Somatostatin receptor subtype-2 knockout mice are refractory to growth hormone negative feedback on arcuate neurons. Mol. Endocrinol. 11:1709-1717.

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